Dosimetric and Toxicologic Assessment of Amphibole Fiber-Containing Material from Libby, Montana

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Introduction and Rationale for Research Projects

The leading source of vermiculite ore for the United States and the world from about 1920 until 1990 was from a mine near Libby, Montana (Horton et al., 2006). The Libby vermiculite ore coexists with a complex array of amphibole mineral types, primarily winchite, richterite, tremolite, and magnesioriebeckite with crystal forms (habits) ranging from asbestiform to acicular/prismatic (Meeker et al., 2003). Occupational exposure to Libby vermiculite is associated with significant increases in asbestosis, lung cancer, and pleural cancer compared to the rest of the U.S. population (Sullivan, 2007). In addition to elevated rates of lung cancer and mesothelioma among Libby residents (ATSDR 2000, 2002), medical testing of persons who lived or worked in the Libby area for at least 6 months before 1991 showed pleural abnormalities (calcifications, thickenings, or plaques) in 17.8% of 6,668 participants (Peipins et al., 2003). Furthermore, exposures to individuals outside of Libby have occurred, and are likely continuing, as asbestos-contaminated vermiculite ore from Libby was shipped to hundreds of locations around the nation for processing, and used as attic insulation in millions of homes throughout the United States. The health effects associated with former and current exposures from the asbestos contaminated vermiculite from the Libby mine continues to be a subject of intensive study and public health concern (Horton et al., 2006). This document outlines a series of research projects aimed at addressing the toxicological effects and dosimetry of amphibole asbestos-contaminated vermiculite from Libby, Montana (referred to here as Libby amphibole or LA).

Although available human data establish the toxicity of asbestos, and preliminary dose-response analyses for LA have been developed, these estimates can be refined by addressing some of the key uncertainties that exist due to gaps in our understanding or lack of quantitative descriptions of internal dosimetry and toxicological effects. This proposed set of projects is aimed at addressing these gaps and providing tools for quantitative characterization, including a comparative analysis of the toxicity of LA relative to asbestos fibers and asbestos-like mineral occurrences. Because the toxicity of inhaled particles and fibers is related to initial deposition patterns and subsequent body burdens of retained internal dose, which is only poorly described by exposure concentration, the research for LA will be framed from the perspective that data are needed to characterize key features which determine inhaled internal dose and the resultant tissue reactions leading to pathogenesis. Other key areas of research will also inform the risk assessment, including inherent toxicity of LA relative to other forms of asbestos and differential susceptibility, including that of different life stages.

Animal data have provided valuable information on the relative potency of different forms of asbestos, and tumor incidence relative to fiber dose appears to correlate with that of humans (Coffin et al, 1992). Animal studies have also yielded the best information available on the pathogenesis of asbestos induced fibrosis and mesothelioma. Few studies have addressed the relative dosimetry of animal compared to humans – a deficiency that will be addressed in these studies.

Dosimetry models provide a versatile tool for computing different internal dose metrics (e.g., retained mass or number). Use of these models for quantitative dose-response analysis can provide critical insights to inform inferences and reduce key uncertainties in exposure-response

analyses of human data. The first phase of the dosimetry project will extend established dosimetry models to describe deposition across a range of fiber sizes. The second phase will extend this model to include mechanisms of clearance including dissolution, mucociliary transport, and translocation. The multi-path particle dosimetry (MPPD) model has been chosen for this development. Simulation exercises using modeled estimates of internal dose can be applied to the existing human data to help improve and refine our understanding of exposure-response relationships, which are based solely on external air measurements at this time. For example, current efforts by EPA Region 8 to develop an RfC from available worker data will benefit from an improved ability to understand and predict health effects based on internal dose. Such predictions can be very informative to hypotheses regarding mode of action (e.g., if mass, surface area or fiber number correlates better with lesions) and can reduce uncertainty by explicitly incorporating mechanistic parameters that dictate internal dose. Dosimetry models also greatly facilitate interspecies extrapolation of laboratory animal experiments with various experimental designs. Due to differences in inhaled dose across species, dose-response relationships for various disease endpoints cannot be accurately predicted based on exposure concentration. Further, toxicology studies can provide mode of action (MOA) information that epidemiological studies can not. For example, toxicology studies can evaluate earlier key events ("precursors") in pathogenesis by providing access to information at various levels of observation (e.g., cellular, biochemical, genomic) and at earlier time points than end-stage outcomes such as mortality and morbidity. Understanding of key events helps to define the disease dimensions that different endpoints in the database represent and is critical to both extending the range of observation as well as to providing mechanistic information needed to translate different endpoints into context. Such a context is requisite for proper integration of toxicological and epidemiological data, rigorous comparative dose-response assessment, and improved estimation of human risk.

Thus the proposed toxicology studies will help define key determinants of internal dose such as *in vivo* clearance rates, provide critical insight on additional key health or pathologic endpoints not evaluated in the human studies, and lay the foundation for construction of a framework that more effectively links exposures to effects. The studies will also be performed in a comparative fashion with respect to different types of asbestos, including fibers not associated with proliferative response to serve as negative controls, in order to ascertain attributes of the pathogenesis and aid understanding of whether LA exhibits differential potency in comparison to other types of fibers.

Although details on the individual components of the proposal are provided separately below, it should be appreciated that this is an integrated and collaborative effort that may not be adequately represented by a sequential list. Certain components will occur in parallel and others rely on iterative development between the experimental research and derivation of parameters or inferences for modeling or assessment. It should also be recognized that the exact mechanism of performing the studies has not been determined in many cases. Many of the studies could be performed either in-house or extramurally. Every attempt will be made during the progression of the studies to incorporate the opinions of experts in the field, and to modify plans based on newly acquired information.

Overall Research Approach

- The National Health and Environmental Effects Research Laboratory (NHEERL) will conduct a series of *in vitro* dissolution and toxicity studies of LA and other fiber types to examine a variety of endpoints, including reactive oxygen species, cytokine production, signal transduction pathways, and chromosomal damage. When integrated with the subchronic inhalation exposure and other *in vivo* studies, such *in vitro* studies offer the potential to compare and examine a variety of other materials more quickly and inexpensively.
- NHEERL will conduct a series of short term comparative toxicity studies through intratracheal instillations of rats and mice, focused on intermittent exposures that are likely to be more representative of actual exposures experienced by residents.
- NHEERL will conduct a subchronic inhalation exposure study in the rat, examining a variety of toxicological endpoints up to 2 years after exposure, and the relationship between duration of exposure and the nature and persistence of effects.
- NHEERL will construct a dosimetry model to refine dose response and dose effect estimates for humans and to facilitate comparisons between humans, rats, and mice. *In vitro* dissolution data will be a key element in those models.

Test Fiber Samples

The primary focus of the research will be on the LA material. Detailed physical and chemical characteristics of LA collected in 2000 were reported by the U.S. Geological Survey in 2003 (Meeker et al., 2003). Sufficient material from the 2000 collection remains and can be used immediately after fractionation to remove non-respirable fibers and particles for planned *in vitro* studies and intratracheal comparative toxicology studies (after due consideration of sample fiber properties required for these studies). Since it is unlikely that sufficient quantities remain for the inhalation studies, new LA material will be obtained from a collection conducted in 2007. This material will be characterized by USGS and used for the inhalation study, and will also be compared to the 2000 sample for the *in vitro* and comparative toxicology studies.

In order to contribute to a broader understanding of asbestos health effects, these research projects will also compare dissolution, *in vitro* toxicity, and *in vivo* toxicological effects of two other fiber samples which are considered by the Agency to have potentially significant environmental health consequences. While the two fiber samples have not yet been selected; they will likely be chosen from among the following: naturally occurring asbestos (NOA; primarily amphiboles), from Region 9 in El Dorado County, California (http://www.epa.gov/region09/toxic/noa/eldorado/index.html); a site-specific chryosotile sample from Region 10 in Whatcom County, Washington (Swift Creek; yosemite.epa.gov/r10/cleanup.nsf/346a4822da38ae7088256da6005fc923/8b0d044466ea186b882572a6006cc71b!OpenDocument) or possibly from California's Clear Creek or Coalinga areas); taconite-associated amphibole from Minnesota; erionite from Dunn County, North Dakota; or other fiber samples including anthophyllite, winchite, or richterite.

The LA and other site-specific samples will be compared to one or more well-characterized samples of asbestos, including: UICC standard chrysotile, UICC standard amphiboles such as amosite or crocidolite, refractory ceramic fibers, relatively inert wollastonite fibers, or glass wool. These comparisons will enable a more complete risk assessment of the inherent toxicity of the site-specific fiber samples. The dissolution and toxicity data of the fibers studied in these projects will be compared with the existing NHEERL fiber characterization database (see Project 1).

In studies where the presence of non-respirable fibers could confound experimental results (*in vitro* and intratracheal instillation studies), samples will be size-fractionated to remove particles and fibers larger than a mass median aerodynamic diameter (MMAD) of 10 microns, which is the median for the respirable fraction of human aerosol sampling. Aerodynamic fractionation will be done by dry centrifugal air separation using "Accucut" or similar established procedures (Graham et al, 1985) and performed under contract. If it is possible to fractionate to even smaller aerodynamic sizes, the resulting size-fractionated samples will be subjected to further analyses of dimensions, biopersistence, and relative toxic potential.

RESEARCH PROJECTS

1. *In Vitro* Dissolution Assays

Goal

The purpose of these *in vitro* studies is to provide data on key physicochemical parameters of clearance mechanisms to refine the dosimetry model predictions of retained dose, which in turn will support the risk assessment. Establishing the dissolution rates and distribution of fiber sizes after incubation with biological fluids will also provide insight on potential pathogenesis and allow relative potency comparisons with similar studies of other types of fibers.

Background

Clearance of deposited inhaled fibers *in vivo* involves a number of inter-related physicochemical and physiological processes: dissolution, leaching, splitting and/or breaking, transport and translocation. Depending on the type of fiber and where it initially deposits, the relative contribution of each of these processes will be different. Additionally, physical and chemical attributes of the fibers such as morphology (fiber dimensions—length, width, height, aspect ratio), mineral habit, effective surface area, porosity, and density will likely influence fiber deposition and ultimate fate *in vivo*. Obtaining data on the physicochemical properties *in vitro* will provide a way to isolate their evaluation and also provide a rapid way to compare the chemistry, and in this case, morphology and mineralogy of different types of fibers. Characterization of these properties can then be superimposed on physical dimensions of the fibers to inform model algorithms when fitting the *in vitro* clearance data.

The purpose of these studies is three-fold: (1) to obtain a rate parameter for LA dissolution, an important physical clearance mechanism used in the fiber clearance model; (2) to provide a dataset specifically for LA that can be used to assess its relative biopersistence compared to other types of fibers and to previous studies conducted both *in vitro* and *in vivo*; and (3) to provide data for quantitative inference and dosimetric modeling of the proposed intra-tracheal instillation and inhalation studies.

Two types of solutions will be compared in the assessment of dissolution, leaching, and splitting/breakage of LA fibers. One solution is a synthetic lung lining fluid (SLF) commonly used in *in vitro* dissolution studies of manmade and naturally-occurring fibrous materials (Zoitos et al., 1997), which has been shown to correlate well with toxicity and to provide useful kinetic information (Hesterberg et al., 2002). The other is an acid solution (HF/HCl/citric acid) used in previous studies conducted by Dr. Philip Cook in NHEERL (Morton et al., 1985; Wilson, 2006). Acid may accelerate physical clearance mechanisms, notably by leaching and breakage, although in a few cases, fiber splitting in the acid leaching assay and in lungs has been observed to counteract net fiber clearance, as well as increase potency. Both the SLF and acid *in vitro* dissolution rates can be calibrated to rates observed in the *in vivo* intratracheal instillation and inhalation studies. For example, in previous studies the acid leaching assay data were calibrated

to *in vivo* data so that one minute of *in vitro* dissolution resulted in fibers of approximately the same dimensions as those resident in the lungs or rats for a year. The *in vivo* burdens were correlated with histopathological lesions. Once calibrated, the *in vitro* system can provide a rapid way to compare key dosimetry determinants that play an important role in fiber persistence and durability.

To monitor dissolution rates we will use three analytical approaches: (1) electron microscopy (both Transmission Electron Microscopy [TEM], supplemented as appropriate by Scanning Electron Microscopy [SEM]) to measure fiber concentrations, dimensions and mineralogy; (2) inductively coupled plasma (ICP) spectroscopy for multi-element quantitative analysis of dissolved tracer elements in the extraction solutions; and (3) gravimetry to measure mass dissolution rate. Together these approaches will confirm and complement each other (Mattson 1994), providing different key dissolution parameters. Electron microscopy is widely accepted as the best method for measuring morphology and mineralogy of all test fibers in this study. The concentration and bivariate size distribution (length, width) of mineral fibers in both exposure and biological samples are the fundamental dose metric data to be provided through electron microscopy. ICP has been used to measure dissolution rates of manmade vs. natural fibers, including asbestos, for both surrogate lung lining fluids (Thelohan et. al. 1994, Scholze et. al. 1987) and acid (Morgan 1997) dissolution studies. ICP and gravimetric analyses can be performed in-house at EPA Research Triangle Park (EPA RTP) to measure total mass.

Comparisons to the Existing NHEERL Mineral and Synthetic Fiber Dose Characterization Database

NHEERL conducted *in vitro* toxicity and dissolution studies and *in vivo* rat toxicity studies of mineral and synthetic fibers from 1978 to 1995 (including, e.g., Coffin et al., 1992, Palekar et al., 1988, Coffin et al., 1983). The NHEERL database of *in vitro* and rat studies has been assembled from archived transmission electron microscopy (TEM) data files and records associated with the toxicity studies. Rats in the toxicity studies were exposed by either intratracheal instillation or by intrapleural injection. The fibers tested include UICC asbestos standards (amosite, crocidolite, anthophyllite, and chrysotile B), and 30 of the samples reported by Stanton et al. (1981) for pleural sarcoma incidence in rats exposed by pleural injection. TEM data for a total of 43 unique mineral and synthetic fiber types are included, with almost all characterized by acid (HF/HCl/citric acid) accelerated leaching for varying time intervals. Nine fiber types have whole lung fiber dose measurements from rats sacrificed at different time periods following intratracheal instillations, and six also have results for kidney fiber dose. A subset of four fiber types also has tissue fiber burden data (pleural membrane, outer lung, inner lung, and kidney) for rats killed 5.5 months after intrapleural injections. The tissue data provide a unique examination of fiber alteration, dissolution, clearance, and transport *in vivo* in association with fiber doses and effects in rats. The basic TEM data, for each particle counted, include mineral and chemical identification, length, width, characteristic fiber cross-section (through limited measurements of average fiber width to thickness ratios), and morphological description (e.g. bundle, clump, plate, etc.). The TEM size and shape data allow calculation of individual fiber surface areas which may be summed to calculate a fiber sample effective surface area. The database represents a total of nearly 1,200,000 fibers measured from a total of 270 individual sample preparations. TEM micrographs and 8x11 glossy photos are cataloged and

provide a visual record of every particle counted and listed in the database. Some additional sample data can be added by counting fibers on TEM micrographs prepared for counting but not completed. This NHEERL database contains some of the best information now available concerning the relative potency of different asbestos fiber types, and potential solutions to problems encountered with measurement and reporting of relative potency. Experimental work to be accomplished in this study will attempt to incorporate new data into the existing database on relative potency of different asbestos types.

Experimental Approach

A characterized sample (i.e., established fiber number concentration, size distribution and morphology) will be provided for each type of test material by EPA Region 8 through an Interagency Agreement with the U.S. Geological Survey. For the purposes of these studies, we define fiber to mean any particle with parallel sides and an aspect ratio > 3.0, regardless of whether the particle originated in asbestiform, fibrous, acicular, prismatic, or other habit. Pilot studies will be performed as necessary to determine unknown variables such as the optimal concentration or mass of LA and other test materials needed (1) to observe incremental changes in tracer element levels following dissolution and (2) to generate appropriate loading for TEM grid or SEM stub preparation. Following the pilot studies, similar dissolution approaches for different types of test materials will be carried out.

Test materials used in the dissolution studies should achieve three goals. First, because *in vitro* studies are a quick and cost efficient means to obtain information to support the dosimetry model and relative potencies, several test materials should be used. These should include as many positive and negative controls for each characteristic being investigated as is feasible. Characteristics of interest include: morphology and size distribution parameters (length, width, thickness, aspect ratio), mineralogy, mineral habit, porosity, splitting potential, biopersistence (or biodurability), and surface area (which may be described as a function of a subgroup of these characteristics). Second, many of the test materials will be used in the NHEERL intratracheal instillation and inhalation studies to allow comparability across the current *in vitro* and *in vivo* work in addition to comparability to previous published studies. Finally, some of the test materials are not intended to assess the toxicity of LA (or lack thereof). Rather, site-specific test materials will be added to determine the relative toxicity of LA to other materials of particular national interest at Superfund sites across the country (see previous Test Fiber Samples section).

Incubation solutions and time course

Incubations will be performed using both SLF and acid solutions. To save on potentially considerable analytical costs, EM analysis may be phased. Once the SLF and acid solutions dissolution tests are carried out, fibers will be appropriately removed from the leaching solutions so that further dissolution of structures does not occur. The fiber structures retained following dissolution of the test materials by both acid and SLF will then be counted and characterized.

Because some of these fibers will not noticeably dissolve over the maximum 2-hour (for acid solution) or 90-day (for SLF) time periods, the longest period for each will be attempted first. If no change is observed in mass, size distribution, and dissolved fiber components, then

shorter incubation periods to establish the time course will not be necessary. If changes are observed, samples will be dissolved for 1, 5, 10, and 90 days in SLF, and for 0.5, 1, 5, 10, 30, 60, and 120 minutes for in acid solution. Longer incubation periods may also be considered. Samples will be removed, prepared for EM on grids, and analyzed.

Electron Microscopy

Samples for EM analysis will be prepared by first removing post-dissolution test material from the cassette, washing or fixing test material to remove residual acid/SLF, putting fixed test material into aqueous solution, filtering aqueous solution onto a 0.1 µm polycarbonate (PC) filter, and then preparing EM filters by evaporative carbon coating. TEM will provide the basic fiber number and size distribution data for characterizing post-dissolution test material, as well as selected area electron diffraction and energy dispersive x-ray identification of individual mineral particles and fibers. This insures data comparability with previous toxicology studies and with the highest quality human exposure data currently being collected at the Libby Site. SEM can provide complimentary data for large particles, including bundles and clumps of fibers, surface morphologies, and quality assurance for uniform particle distribution on filters. EM sample preparation/fixing and EM filter preparation support shall be provided by EPA RTP. EM analytical support shall be provided by EPA Region 8's laboratories, currently under contract to support analysis at the Libby Site once that portion of the study budget is transferred to EPA Region 8.

Specific Aim 1: Establish a fiber dose – cancer relative potency model and reanalyze Stanton's studies from the existing NHEERL database**.**

The existing NHEERL database will provide the foundation for a peer-reviewed journal article on an *in vivo* fiber dose – cancer relative potency model for amphiboles. This paper will include intratracheal and intrapleural rat toxicity effects, tissue dose measurements, and accelerated acid leaching data. A second journal article will perform a reanalysis of Stanton's studies of the relationship of fiber dimensions and carcinogenicity (Stanton et al., 1981) to provide further development of relative carcinogenicity factors based on length, width, durability, and surface area at the most relevant post-exposure time with respect to development of pleural sarcomas. Longer term applications of the database may include: (1) application of the amphibole relative potency model to LA or other site-specific samples; (2) investigation of fiber clearance and translocation behavior in rats; and (3) use of the database for dose calculations and dose-response comparisons to develop a toxicity assessment for LA, presuming the availability of compatible TEM data from new studies.

Specific Aim 2: Determine rates of acid accelerated leaching (AAL) of fibers.

NHEERL research in the 1980s developed a method using an acid (HF/HCl/citric acid) solution to rapidly assess the relative durability and potential for shape/size alteration of mineral and synthetic fiber samples utilized in rat exposures. It was hypothesized that the resultant fiber characteristics could be relevant to cancer potency determinations. Although the purpose of the AAL method was not to directly measure rates of dissolution relevant to biological fluids, the changes in fiber numbers and sizes/shapes in the acid, typically for time periods ranging from

less than ten seconds to a few minutes, could be empirically related to changes in fiber characteristics observed for up to two years residence in rat lungs. The TEM data for some 30 different fiber types characterized with the leaching method are available through the database of archived NHEERL sample characterization data described previously. The fiber sample data include UICC standard fiber samples used in many animal studies reported in the literature. Thus, new fiber samples characterized with the AAL method can be compared to the 30 different fiber types presently in the data base and potentially many reported effects data related through a standard set of high quality fiber dose characterizations. AAL testing will be performed on all study samples. Gravimetric and elemental component dissociation rate constants will be calculated and filters submitted for EM analysis, which will document particle size distribution (PSD) changes in morphology and mineral form. Work will begin in the fourth quarter of 2007, and reported in the second quarter of 2008.

Specific Aim 3: Determine rates of fiber leaching in synthetic lung lining fluid (SLF).

SLF leaching rates are slower than that of the proposed acid mixture, and more commonly used for the relatively fast-dissolving test samples such as glass wool. However, the SLF method has frequently been applied to asbestos materials to demonstrate their much slower (typically 300 – 1000X) dissolution rates. SLF leach results can also be directly compared to those from scores of other studies using this same leaching fluid, and compared to the accompanying *in vitro* and *in vivo* toxicology studies. The SLF leaching apparatus will be constructed, and tests performed on all study samples. The gravimetric and elemental component dissociation rate constants will be calculated and filters submitted for EM analysis, which will document particle size distribution (PSD) changes in morphology and mineral form. The SLF project will take longer to finish compared with the AAL, since the methodology is more complex and the time points are longer (90 days) compared to AAL. Work will begin in the fourth quarter of 2007, and reported in the third quarter of 2008.

Specific Aim 4: Analyze the elemental content of leached material**.**

Gravimetric analysis of the amount of material remaining on the filter from the leach tests will be performed on a calibrated, externally audited 5-place balance, and reported on the basis of soluble mass fraction of the original sample. We will perform elemental analysis closely following EPA Method 200.7 rev4.4 on a PerkinElmer 4300DV ICP-OES. This setup has sufficient sensitivity to quantitate all of the major and minor chemical constituents of each fiber. Calibration and QC standards will be prepared in matrix-matched solutions of each leach fluid, and quantitation accuracy demonstrated by the method of standard additions. Total elemental analysis of the parent materials will be performed after complete dissolution in a microwave digester, in order to report dissolution test results on the basis of soluble mass fraction of the original sample. The gravimetric and elemental analysis will be integrated with the EM results from the first two specific aims, and the results will be compared and contrasted with previous literature for all study sample types. Work will begin in the fourth quarter of 2007 and reported by the second quarter of 2009.

Specific Aim 5: Determine fiber-induced oxidation using Oxygen-18**.**

Oxidation occurs at the epithelial surface of the respiratory tract as a result of asbestos exposure. The extent of this oxidation appears to be a highly important predictor of the fibrogenicity of fibers – assuming they are not degraded first. One of the most effective ways to measure this oxidation is through exposure of SLF containing asbestos to an atmosphere where normal oxygen (oxygen-16) is replaced with heavy oxygen (oxygen-18). Other measurements exist for this oxidation, but none are as free from sample preparation artifacts as the oxygen-18 incorporation. The method for quantifying oxidation as oxygen-18 incorporation in a type of SLF different from that used by Zoitos (1997) has been reported (Sun et al, 2001). LA will be compared with other test materials in the ability to oxidize SLF and this will be compared with the previous work published by Sun and coworkers (2001). Work will begin in the fourth quarter of 2007, and be reported by the second quarter of 2009.

Project Resources

To accomplish the principle objectives of completion and submission of manuscripts related to the NHEERL database, $60,000 in contract funds will be provided for statistical analysis and database support for the period of September 2007 – February 2008. The database project will require effort of 0.3 Principal Investigator (PI) FTE (Cook). Pilot studies of AAL and SLF, and materials for ICP-OES analysis will cost $10,000, and the SLF dissolution study equipment will cost $24,000. The costs for sample preparation and EM characterization are estimated at $2,000 per sample; a total of $270,000 in these costs are anticipated. ICP-OES analysis for 20 elements for each sample of AAL and SLF solutions will take at least 12 weeks to set up, run, and prepare reports. To assist in tissue preparations for EM, elemental analysis, and oxygen-18 studies, funds will be provided to pay two Senior Environmental Employee Program (SEE) employees half salaries each for two years (B. Crissman and J. Sullivan; total 1.0 FTE/year = $50,000/year for each of 2 years). This project will also require EPA FTE support from the following individuals (total effort per year for 2 years total): PI: 0.25 Hatch, 0.15 Jarabek; Technical: 0.25 K. Crissman, and 0.25 McGee.

2. Use of *In Vitro* Toxicology to Compare the Potency of Different Test Materials

Goal

The purpose of these assays is to compare the ability of asbestos obtained from several sources to cause significant biological effects in cultured cells. The effects studied will be compatible with effects studies in animal toxicology studies. The *in vitro* approaches using human cells will focus on respiratory tract epithelial cells and macrophages because these are the cells that first come in contact with inhaled substances such as asbestos. These *in vitro* studies are a rapid, inexpensive way to compare the relative potency of many different types and sizes of fibers and inform the design of animal instillation and inhalation studies. They will also be able to provide information about predictive and clinical biomarkers, and the mechanisms by which different asbestos fibers cause toxicity. Resulting data may provide information relating to mode of action and dose metric, both of which may inform the risk assessment approach.

Background: Cell Culture Systems

Epithelial Cells

Dosimetry studies have shown that nearly all particles are deposited between the large airways and the end of the tracheobronchial region, before the alveoli themselves. This is important because the epithelial cells that populate the alveoli are different from the epithelial cells that populate the airways and tracheobronchial tract. For these experiments, primary human airway epithelial cells will be used as opposed to cell lines, because of concerns about how well the response of transformed cells mimics that of primary cells. Such primary cells have been exposed to a wide variety of particles for more than a decade by NHEERL investigators and have been shown to be a sensitive and reliable indicator of response. More importantly, biological changes in these cultured cells following exposure to particles *in vitro* have been shown to be similar to biological changes in airway epithelial cells after instillation of the same particles into rodents. These cells are routinely available through brush biopsies taken during bronchoscopy of human volunteers as part of the EPA Human Studies Division (HSD) controlled air pollution exposure study program. In addition to obtaining cells from normal healthy individuals, it is also possible to obtain cells from potentially susceptible populations such as those with asthma or those with specific genotypes that may render them susceptible to particles (e.g. null for anti-oxidant genes such as GSTM1).

Alveolar Macrophages

The function of these cells is to remove foreign substances (e.g. bacteria, particles) from the lung by phagocytosis and to act as the first line of host defense in the lung. Previous studies by NHEERL investigators have shown that these cells interact extensively with particles, including asbestos fibers. Primary human alveolar macrophages are routinely obtained from volunteers during bronchoscopy. These cells do not divide, but can be maintained in culture for 24 - 48 hrs. *In vivo*, there is undoubtedly communication between macrophages and epithelial cells. By co-culturing these two cell types *in vitro* it is possible to duplicate some of this intercellular communication.

Mesothelial Cells and Fibroblasts

The effects of fiber samples on mesothelial cells and fibroblasts may also be determined as resources permit, as these are progenitor cells which are key in the development of mesothelioma and fibrosis, respectively.

Endothelial Cells

Endothelial cells line blood vessels, and reside only a few microns from airway epithelial cells. Current research with ambient air pollution particles suggests that if particles leave the lung, endothelial cells become primary targets. Endothelial cells play a key role in mediating vascular tone (ability of blood vessels to contract or dilate as needed) and blood coagulation/clotting - both intimately involved in the potential formation of blood clots. Endothelial cells also play a significant role in controlling the level of vascular inflammation. NHEERL scientists have previously studied the effects of various particles on cultured human endothelial cells. However, these cells would not be used in these studies unless there is convincing data that LA fibers were translocated from the lung to the vascular system.

Experimental Approach:

Cell Exposure

Varying concentrations of asbestos fibers will be applied to cultured epithelial cells, macrophages, fibroblasts, mesothelial cells, or endothelial cells to obtain a dose-response curve and to ensure that non-toxic levels of fibers are being used. At different intervals following exposure, cell supernatants will be removed and stored for analysis of various mediators described below. RNA will be isolated from cells for RT-PCR analysis of changes in mRNA expression for mediators described below, and for micro-array studies. Confocal microscopy will be used on some cultures to measure viability and oxidative stress induced in cell organelles. Cell uptake of fibers will be assessed by TEM.

Tiered and Comparative Toxicological Assessment of Mechanisms of Injury and Mode of Action (MOA) of Mineral Fibers

In vitro toxicological test methods have historically provided key information relating physicochemical properties of microscopic particles to toxicity as well as mechanisms of injury and mode of action. Previous work of NHEERL investigators has assessed the effects of various particles on respiratory tract cells using the following tiered approach:

1. Toxicology Assays. Cell viability and damage will be monitored by well established assays such as trypan blue exclusion or LDH release, and with newer fluorescent dye assays coupled with confocal microscopy that are capable of measuring intra-cellular damage of cell organelles. Since epithelial cells and alveolar macrophages initiate a pulmonary inflammatory response to many inhaled substances, the ability of asbestos fibers to stimulate the release of a number of pro-inflammatory and pro-fibrotic cytokines (e.g. TNF, IL-1, IL-8, IL-6),

prostaglandins and leukotrienes, will be monitored, as will the production of indicators of oxidative stress (e.g. heme oxygenase). Confocal microscopy will be used to assess intracellular oxidative stress in the cytosol as well as specific target organelles such as mitochondria and the nucleus.

The comet assay will be used as an initial screen to determine if fibers will induce DNA damage in lymphocytes. If this is positive, then concentration-response curves using various dose metrics (e.g., fiber number or surface area) will be constructed. If negative, other cell types or cell lines, especially macrophages or mesothelial cells will be investigated. Specific properties of the fibers responsible for the induction of DNA damage will be investigated.

2. Cell Function Assays. Macrophages are the lung's primary defense against inhaled micro-organisms. Many inhaled toxicants interfere with the ability of macrophages to kill micro-organisms. The ability of mineral fibers to modulate macrophage function by altering phagocytic capability and production of reactive oxygen species will be assessed. Epithelial cells act as a barrier between the outer airways and the vascular system; they also form part of the mucociliary escalator which clears particles from the airways. Inhaled particles are known to affect both of these processes. The ability of mineral fibers to alter mucin production and loss of cilia in primary differentiated cells will be characterized, as will potential damage to tight junctions that are present between the cells.

3. Cell signaling pathways. The synthesis of many pro-inflammatory mediators or indicators of oxidative stress is controlled by a number of MAP kinase signal transduction pathways (e.g. ERK, JNK, p38). Our previous work in which cultured cells are exposed to a wide variety of particles has indicated that while many different particles can stimulate the production of pro-inflammatory markers or indicators of oxidative stress, they do so by different mechanisms and through the induction of different cellular signaling pathways. Signaling typically involves initiation and propagation of phosphorylation events that occur on specific amino acid residues present on signaling intermediates. The level of phosphorylation is regulated by the opposing activities of kinases, which phosphorylate proteins and that of phosphatases, which dephosphorylate them. NHEERL investigators have shown that particles and fibers can disrupt phosphohomeostasis by inhibiting the activity of phosphatases and thereby promoting the accumulation of phosphorylated forms of pivotal signaling intermediates. The net result is increased levels of phosphorylated signaling proteins and, therefore, an elevated signaling tone leading to a raised level of cellular activity and exaggerated cellular responses. Therefore, the ability of different types of mineral fibers to activate or repress different signal transduction pathways will be assessed.

4. Gene Expression Profiling (Micro-Array). The ability to monitor particle-induced changes in the expression of 40,000 genes simultaneously is a powerful tool with the potential to identify biomarkers of exposure, effect, or sensitivity unique to individual particle types. We have shown that exposure of lung epithelial cells to different types of particles results in changes of several hundred different genes. In addition to a number of genes which are activated by nearly all particles, each particle we have tested also activates a unique set of genes. Assigning these differentially regulated genes to specific cell pathways and functions has allowed us to link specific particles with the activation or repression of these pathways and processes. This is

likely the most powerful approach to determining the comparative potency of LA with other types of asbestos and particles.

Project Resources

A postdoctoral fellow will be recruited to culture the cells and perform toxicology, signal transduction, and micro-array assays ($75,000/year, $225,000 total). A student contractor will be needed to perform DNA and chromosomal damage assays ($30,000/year, $60,000 total). Funds to purchase supplies and provide EM analysis of samples ($50,000/yr, $150,000 total) will be provided. This project will also require EPA FTE support from the following individuals: 0.5 total FTE from partial time of principal investigators (R. Devlin, A. Kligerman), and 0.6 total FTE from technical scientists (each per year for 3 years total).

3. Comparative Toxicology in Rats

Goal

The purpose of this series of intratracheal instillation studies is to provide mechanistic understanding of the comparative toxicity of different types of fibers, fiber-translocation kinetics, and age and disease-related susceptibility *in vivo*. Data obtained from these studies will be used to support the risk assessment and provide information regarding comparative toxicity of different fiber types. The use of single versus multiple episodic instillations will allow one to determine early biomarkers of late fibrogenic and tumorogenic effects and fiber-specific mechanisms of inflammation or oxidative stress that lead to chronic lung pathology. Although intratracheal instillation delivers material to the respiratory tract in a non-physiologically relevant high dose rate, this technique may provide comparable toxic effects to inhaled pollutants. This approach will allow one to test multiple materials at varying concentrations simultaneously. Further, intratracheal instillation studies will allow precise dose administration of each test material in the respiratory tract, and thus, provide accurate determination of comparative biological effects that are not influenced by differential fiber deposition following inhalation. Additionally these studies provide important data on parameters for refining clearance rates used in the dosimetry model, notably data that can be used to estimate physiological components of transport and translocation, thus supporting the risk assessment. Initial rate estimates for these clearance mechanisms will serve to refine the model algorithms and additionally be used to compare to rates observed in the inhalation studies. Rats are proposed because they are the species most tested in the extant database. Use of mice is proposed to inform considerations of comparative species sensitivity, as well as to allow potential for use of genetically defined lines for future study.

The studies are listed in order of priority. We will definitely complete Specific Aims 1 and 2. Specific Aim 3 will be completed in conjunction with the dosimetry studies described in Section 5. We will assess the the susceptibility of neonatal rats, as resources permit, in Specific Aim 4. If limited time and resources permit, we will study rat and mouse models of susceptibility in Specific Aim 5.

Experimental Approach

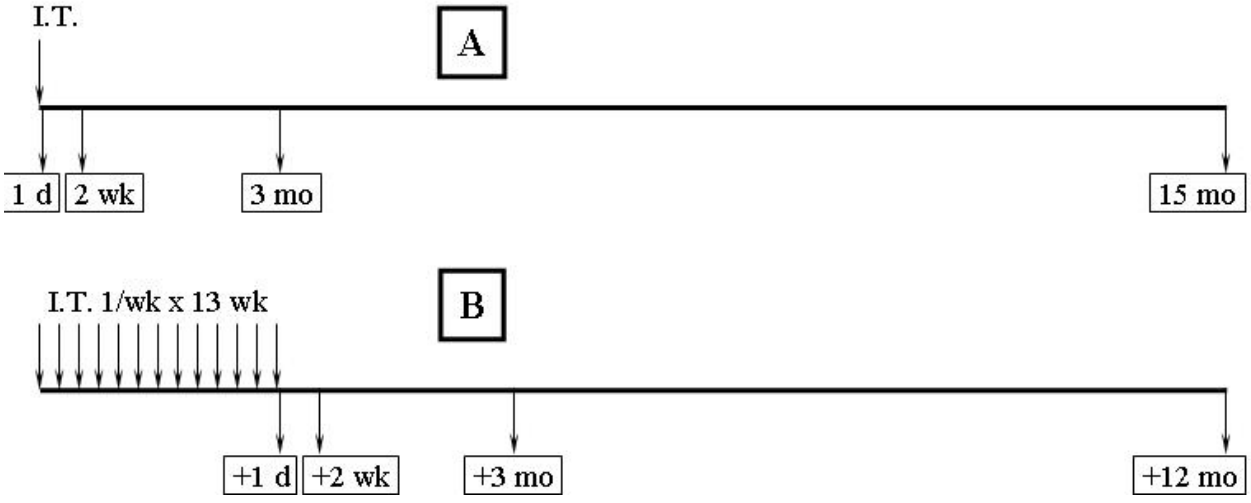
We will compare the toxicity of LA with positive and negative fiber sample controls administered by intratracheal instillation (or a comparable technique, oropharyngeal aspiration) in rats. We will assess dose-response and time-course relationships and the effects of repeated intermittent dosing. If resources and time permit, these projects will include assessments of juvenile susceptibility (through administration of fibers to neonatal rodents or through administration of fibers to pregnant rodents), and models of susceptibility and autoimmune effects. All of these effects will be correlated with physical and chemical characteristics of the test materials (pre-instillation and recovered after tissue digestion) and related back to the existing NHEERL database.

Specific Aim 1: Determine the dose-response relationships of LA in rats.

In order to systematically evaluate the toxic potency of a given substance to support risk assessment, it is important to identify concentration-dependant effects in acute and long-term exposure scenarios. Using acute and episodic long-term exposure scenarios, these studies will also focus on identification of early biomarkers of chronic effects. Markers of fibrosis (lung weight and hydroxyproline), apoptosis, oxidative stress, DNA damage, and cell proliferation will be analyzed to understand the relationship between acute and chronic effects.

Young adult rats will be exposed by intratracheal instillation to saline alone or LA suspended in saline at 4 different concentrations either once or once per week for a total of 13 weeks (see Figure 1 below). Pulmonary injury (BAL fluid protein and cytokines) and oxidative stress biomarkers will be analyzed 1 day, 2 weeks, 3 months, and 15 months post-exposure in the single exposure protocol. In the long-term episodic exposure, necropsies will be performed 1 day, 2 weeks, 3 months, and 12 months post-exposure in rats, and measures of pulmonary inflammation, oxidative stress, and pathology will be determined. Although not shown in Figure 1, we will also hold groups of rats for up to 2 years after instillation in order to determine if fibrosis, mesothelioma, or lung tumors develop. The endpoints will include pulmonary and pleural inflammation and injury markers (macrophages, neutrophils, lymphocytes, mast cells, protein, lactate dehydrogenase (LDH), N-acetyl-β-D-glucosaminidase (NAG), γ-glutamyl transpeptidase (GGT)), lung and pleura pathology, gene expression analysis for markers of oxidative stress, nuclear DNA damage, and apoptosis markers. Selected tissues will be analyzed by TEM to determine fiber burden at different times after exposure.

Figure 1. Timelines showing intratracheal instillation (I.T.) and sacrifice times (in boxes) for rats exposed to LA using 2 different protocols: (A) single instillation, and (B) multiple instillations equivalent to length of subchronic exposures in Section 4. Both protocols apply to Specific Aim 1 and Specific Aim 2. Some groups of rats will be held for up to 2 years after instillation (not shown).



These concentration-response and episodic exposure data will provide information on concentrations that would cause toxicity, fibrosis, lung cancer, or mesothelioma in rats. Since mesothelioma and lung cancer will typically require longer than 1 year to develop, we will hold some groups of rats for up to 2 years after exposure in order to assess possible tumor development (not shown in Figure 1). These studies will also provide insight into consideration

of LA exposure doses for comparative analysis with other fibers. Since the disease development process occurs over several months following exposure in humans, these studies will also provide information about early biomarkers and the time course of inflammatory changes in the lung following LA exposure.

Specific Aim 2: Compare the toxicity of LA relative to other test materials.

While other types of asbestos fibers have been shown to cause differential toxicity and carcinogenic potency, it is not known if LA fibers are more toxic than other site-specific samples or asbestos standard samples. Physico-chemical properties, and therefore the toxicity and carcinogenic potency, are also likely to be different among different fiber types. Comparative evaluation of acute effects of one instillation versus multiple instillations may allow identification of acute biomarkers that indicate chronic fibrotic changes for multiple fiber types. Thus, it may be possible to estimate relative inflammatory effects of different fibers in acute exposure scenarios and how they relate to long-term fibrosis, lung cancer, or mesothelioma development. These studies will provide mechanistic information about differential acute inflammatory effects in rats and how that may relate to later development of mesotheliomas.

Young adult male rats will be exposed intratracheally to saline or different types of test materials at previously determined dose levels based either on previous studies in the NHEERL database or on the dose-response studies described in the previous specific aim, and acute effects will be determined at the time points indicated in Figure 1A following exposure. Rats will also be exposed to saline or different test materials intratracheally, once per week for 13 consecutive weeks and analyzed at the time points indicated in Figure 1B post exposure (as well as up to 2 years after instillation). The same endpoints described above for Specific Aim 1 will be evaluated in this specific aim. Blood plasma will be stored for analysis of the presence of autoimmune antibodies and total IgA. This exposure regimen will allow identification of fiber-specific differences in acute and long-term effects including inflammation, mesothelioma, lung cancer, and pulmonary or pleural fibrosis.

The group of rats sacrificed 2 years after exposure will likely provide data on mesothelioma induction, based on previous studies (Coffin et al, 1992) with the most potent materials. Tumors will be removed at the time of death or severe morbidity and the remaining fibers in the lungs and pleura quantified as done previously to provide a denominator for the expression of tumor potency. The retained fibers in the tissues within the lesions will be quantified and compared eventually to data on fibers within similar tissues obtained by EPA Region 8 investigators from exposed human autopsy samples. The evaluation of the fibers in tissues within lesions will allow inferences to be made about the types of fibers most connected with the pathology, and whether fiber splitting, dissolution, or other changes have taken place in these fibers during the course of the pathogenesis. The overall comparative analysis of fiber toxicity in rats will be important in determining the relative toxicity of LA fibers compared to other fiber types tested in these studies and in the NHEERL historical database.

Specific Aim 3: Determine if LA translocates to extrapulmonary organs in rats.

To provide time course data with which to characterize translocation rates, rats will be exposed to concentrations that correspond to those chosen for Specific Aim 1 above. Animals

will be sacrificed immediately after instillation, and at 1.5, 3, 6, and 24 hrs and 1 week after instillation. Tissue samples of the trachea, lungs, and GI tract will be taken and frozen at -20 C. These samples will subsequently be ashed and submitted for EM analysis, which will document particle size distribution (PSD) changes in morphology and mineral form as previously described for the *in vitro* dissolution studies. Urine samples will be collected during the week following instillation and also analyzed for fibers.

Specific Aim 4: Evaluate whether neonatal rat intratracheal instillation exposures are associated with increased injury, impairment of lung growth, or increased sensitivity to develop tumors.

Due to differing rates of metabolism and mechanisms of exposure, children may be at increased risk from the effects of environmental asbestos exposure relative to adults. In addition, developing immune and maturing organ systems are likely to be more vulnerable than fully developed systems to asbestos-induced acute and chronic health effects. Rodent studies can be used to evaluate the long-term effects of neonatal exposure since within a shorter life span, one can perform asbestos exposures and determine incidence of disease over their adulthood and senescence. To evaluate these potential effects, neonatal rat pups will be intratracheally instilled once per week beginning at 1 week of age, for 13 consecutive weeks and later analyzed for the presence of lung inflammation, fibrosis, and possible mesothelioma along with evaluation of molecular markers of apoptosis and oxidative stress. These analyses will be done at 1 week or 4 weeks post instillation of saline or LA fibers. These studies are critical in determining susceptibility of young versus adult rodents tested under Specific Aims 1 and 2.

It is possible that upon maternal pulmonary exposure some of the asbestos fibers translocate to extrapulmonary sites including the developing fetus. Translocation of asbestos fibers to the circulation and the fetus has been previously demonstrated (Haque and Vrazel, 1998; Haque et al., 1992). In order to further address the role of direct fiber effect on the fetus versus a secondary developmental abnormality as a result of pulmonary inflammation, we propose an experiment which will allow a sensitive analysis of extrapulmonary tissue fiber burden following pulmonary exposure. If resources permit, timed pregnant female rats will be intratracheally instilled once every three days for a total of five times with LA fibers. Urine samples will be collected daily for three days prior to necropsy. One day prior to delivery, rats will be deeply anesthetized and lung, blood, intestine, pleura, spleen, kidney, placenta and fetuses will be preserved in filtered formalin, and analyzed for asbestos fibers. A total of five asbestos exposed plus two control rats will be used for TEM analysis. Two to three fetuses per dam will be analyzed for translocation of asbestos fibers. Concurrent exposures of extra rats will be done to determine pathological impact of fibers in different tissues at later time points for which fiber burden is determined.

Specific Aim 5: Evaluate whether underlying genetic susceptibility to develop diseases predisposes rats and mice to exacerbated adverse effects of LA.

Although the exposure to LA fibers has been associated with a marked increase in incidence of mesothelioma in both mine workers and the general population, not all individuals are equally susceptible to developing a disease. It is not known which human subgroups may be more vulnerable to LA-induced mesothelioma. Previous studies have shown that humans with

cardiovascular diseases are more susceptible to particulate air pollution-induced mortality and morbidity. We and others have shown that animal models which demonstrate susceptibility to hypertension or atherosclerosis are more susceptible than healthy rodents to ambient and combustion source particle-induced inflammation and oxidative stress.

There are wide strain variations in development of fibrosis and tumors. Acute inflammatory response in different strains of rats and mice, especially those having underlying genetic disease may be more severe than the response in healthy animals. In order to consider the use of highly sensitive species for risk extrapolation, we need to determine the relative susceptibility of LA fibers on normal healthy and disease-prone rat and mice strains. We propose to use healthy Wistar Kyoto (WKY) and spontaneously hypertensive (SH) rats and healthy and atherosclerosis-prone Apo E knockout mice for determination of the mechanisms of enhanced susceptibility to pulmonary and systemic inflammation, oxidative stress, and mesothelioma.

Rats (SKY and SH) and mice (normal and Apo E knockout) will be instilled once or 13 times (once/week) to saline or LA fibers and a variety of pulmonary and systemic markers of inflammation, oxidative stress and DNA damage described in the prior specific aims will be assessed. These studies will provide insights into potential biological mechanisms of susceptibility.

Project Resources

A postdoctoral fellow ($75,000/year for 3 years) and a graduate student ($40,000/year for 2 years) will be funded to conduct comparative toxicity tests in rats. A SEE employee will also be funded ($50,000/year) for 2 years. To carry out a systematic comparison of LA to two other site-specific test materials, an additional $210,000 will be provided. Funds will also be provided for animals ($70,000), gene chips ($50,000), other lab supplies ($87,500), pathology contract support ($37,000), and TEM sample preparation and analysis ($200,000). FTE costs are estimated at 0.6 PI total per year (S. Gavett and U. Kodavanti to mentor postdoctoral fellow and student and to guide completion of studies) and 1.75 technical scientists per year for gene chip work and assistance in conduct of studies).

4. Inhalation Toxicology in Rats

Goal

This project provides data on the relative potency of inhaled Libby amphibole (LA) compared to UICC amosite, a known fibrogenic and carcinogenic amphibole asbestos fiber. In addition to providing information on the intrinsic toxicity of LA, these inhalation studies provide data on the inhalability of LA and its initial deposition distribution which is necessary for refined dosimetry parameters (e.g., transport and translocation rates) and accurate retained dose predictions. Data obtained from these studies will support the risk assessment and identification of potential biomarkers.

Background

To accomplish the direct comparison of toxicity of LA fibers to amosite, we will conduct a subchronic 90-day nose-only inhalation exposure of male Fisher 344 rats followed by no-exposure recovery periods up to one year post-exposure (up to 2 years if resources permit). This study will provide dosimetry and toxicity information on fibers used in physiologically relevant inhalation exposures, with intensive measures of fiber burdens, clearance, pathology, and recovery. This study will provide key information in a timelier manner than a full two year chronic inhalation study, which has been nominated for study and is likely to be conducted by the National Toxicology Program sometime in the future. The NTP two year chronic study will also provide valuable information for the risk assessment at Libby, especially with respect to tumorigenic potential in rats, but it is likely that it will take 5 years for that study to be completed and fully analyzed. The subchronic study described here will be conducted most efficiently through an outside contractor located near EPA's National Health and Environmental Effects Laboratory in Research Triangle Park, NC.

Experimental Approach:

Specific Aim 1: Determine the proper concentration of LA fibers to use for the subchronic inhalation study in a 2-week range-finding exposure of rats.

Preliminary studies of test materials will be conducted to characterize composition and aerosol properties in a flow-past nose-only inhalation exposure system. The system may include Cannon type units or stackable tiers (RCC, Füllinsdorf, Switzerland) each accommodating 16 nose-only exposure tubes (Bernstein et al., 2006; Bermudez et al., 2003). Special safety measures for handling asbestos contaminated materials will be used for containment, handling, and disposal, including waste water. Aerosol concentrations will be determined during exposures with RAM monitors and final mass concentrations determined gravimetrically, and particle and fiber size distributions with concentrations (fibers/unit mass of aerosol) will be determined by TEM. To determine the appropriate fiber concentrations to use for the subchronic study, a range-finding study will first be conducted in which rats will be exposed 6 hours/day, 5 days a week, for 2 weeks to LA at 5- to 10-fold differences between each of 3 concentrations (as determined by mass concentration and thus associated fiber numbers). Endpoints examined from the 2 week study (see below) will guide the choice of concentrations to use for the subchronic

exposure study to follow, which will be targeted at 2- to 3-fold differences between each of 3 concentrations. In the 2 week range-finding study, rats will be exposed nose-only to air, one of the three concentrations of serially diluted LA aerosol, or amosite asbestos as a positive control (20 rats/group; 100 rats total). All rats will be sacrificed one day after the final exposure. The critical endpoints to examine as a tool for determining concentrations to use in the subchronic assay may include inflammatory cell numbers in the bronchoalveolar lavage fluid, including neutrophils, or epithelial cell proliferation. This range-finding study will also provide data on the initial deposition distribution of LA. For this purpose, fiber burden analysis will be conducted by TEM as described previously for the *in vitro* dissolution study. Extra rats will be sacrificed immediately after the first day (6 hr) exposure and after 5 days of exposure, and the following tissue samples taken for analysis: nasal airways (upper respiratory tract; URT), larynx, trachea, intestine, pleura, and lung lobes.

Specific Aim 2: Evaluate the relative toxicity of LA fibers in comparison to amosite asbestos in a 90-day subchronic exposure of rats.

A report of the ILSI Risk Science Institute Working Group (Bernstein et al., 2005), which provides recommendations for fiber concentrations, times of exposure, and endpoints to examine in subchronic inhalation testing of fibrous materials, is proposed as a starting point for the design of the 90-day subchronic study. A sample paradigm for the conduct of this exposure is provided in a recent 90-day subchronic study of Brazilian chryosotile asbestos (Bernstein et al., 2006). The ILSI Working Group recommends that the target high concentration in a 90-day protocol should be 150 fibers/cm^3 with lengths greater than 20 μm (Bernstein et al., 2005), although this concentration of long LA fibers may not be practically achieved; the actual concentrations of fibers and their distributions remains to be determined based on analysis of the aerosol concentrations observed in testing. In the Brazilian chryosotile asbestos study, a high concentration of 8941 total fibers/cm^3, 207 fibers > 20 μm/cm^3, and a mass concentration of 3.56 mg/m^3 was achieved (Bernstein et al., 2006). In any case, total mass concentration will be documented daily, and the concentrations and dimensions of the entire fiber size distribution will be measured on a weekly basis. Reporting will also include the fractions of WHO fibers (> 5 μm length), and fibers > 20 μm length measured on a weekly basis.

Inhalation exposure of male Fisher 344 rats will be conducted in 5 exposure groups: 3 concentrations of Libby fibers (concentrations to be determined by the range-finding study which are not more than 3-fold differences between each concentration), a zero (air) control, and a positive control (UICC amosite at the same total fiber concentration as that of the high concentration LA group). A negative control such as wollastonite fibers was considered but it was determined that the expense of running this control would be better used in more intensive measures of responses in the LA, air, and positive control groups. Animals will be assessed for all relevant clinical observations including body weight at least at weekly intervals, and will be screened for the presence of common rodent pathogens according to standard protocols in both the range-finding and subchronic studies. In the subchronic study, animals will be exposed 6 hours/day, 5 days/week, for 13 weeks and will be sacrificed at 5 time points (after 1 and 3 months of exposure, and 3, 6, 12, and 24 months post-exposure). Rats held up to 2 years after exposure will enable us to assess the development of tumors. The post-exposure periods are included to evaluate progression, persistence, or recovery of key parameters. Twenty rats from

each of the 5 groups will be assessed for a number of endpoints at each of the 5 time points (see below; 100 rats per time point, 600 rats total). The contractor will conduct all exposures, sacrifices, and coordination of pathology with a selected subcontractor. The contractor will prepare tissues for fiber burden analysis, but TEM analysis of tissue fiber burden will be conducted by a separate contractor. Dosimetric modeling of fiber clearance will be conducted within EPA.

Endpoints Examined in Both Specific Aims

Histopathology, plasma endpoints, bronchoalveolar lavage (BAL), pleural lavage fluid (PLF), and tissue fiber burden will be assessed in all groups. Since these three mutually exclusive measurements require analysis of the same lung tissue, the endpoints for each will be assessed in separate groups of rats. Accordingly, of the 20 rats in each exposure group, 7 will be used for assessment of histopathology and proliferation, 7 will be used for assessment of inflammation in BAL and PLF, and 6 will be used for assessment of fiber burden.

Histopathology and Cell Proliferation. In the first group, proliferation will be determined by surgically implanting rats with mini-osmotic pumps filled with 10 mg/ml 5-bromo-2'-deoxyuridine (BrdU) 3 days before killing. Lungs and pleural tissues from these rats will be fixed with formalin and stained with hematoxylin and eosin or trichrome, for pathological indication of fibrosis, and BrdU-labeling as an index of epithelial proliferation will be assessed.

Bronchoalveolar lavage (BAL) and plasma endpoints. In the second group of rats, both lungs and pleurae will be lavaged with 30 ml/kg body weight of PBS, and total and differential cell counts, total protein, lactate dehydrogenase, beta-glucaronidase, N-acetyl-β-D-glucosaminidase, and alkaline phosphatase will be determined. Plasma will be tested for the presence of autoantibodies and IgA, as in the comparative toxicology studies.

Fiber burden. In the third group of rats, animals will be sacrificed and the URT, larynx, trachea, lung lobes, intestine, and pleural tissues will be freeze-dried, ashed, resuspended in ultrapure water and analyzed by TEM as described above for the *in vitro* dissolution studies. The numbers of tissue samples to be analyzed for fiber burden depends on predictions of fiber burden from preliminary modeling efforts and the exact costs which remain to be determined. Quality Assurance will be conducted according to contractor and EPA standards.

Project Resources

The estimated cost for this project is $1,112,000, which includes costs for the contract to carry out the asbestos inhalation exposures and most endpoints listed above, plus a separate contract to conduct TEM analysis of fiber burden in lungs and other organs (estimated at $200,000). Additionally, EPA will provide FTE support for contract supervision and carrying out some endpoints, estimated at a total of 0.5 FTE per year for 2.5 years.

5. Dosimetry Model Development and Simulation Studies

Goal

The purpose of this project is to develop a dosimetry model to predict fiber deposition and retained fiber burden in rodents (rats and mice) and humans. This model will be developed and verified with data specific for LA, and can be used to estimate different dose metrics for refinement of dose-response relationships used in risk assessment.

Background

Dosimetry models such as the one developed by the International Commission on Radiological Protection (ICRP, 1994) and the multi-path particle dosimetry (MPPD) model that estimate particle deposition, clearance, and retention have proven especially useful to the process of setting the National Ambient Air Quality Standard (NAAQS) for particulate matter (PM) (US EPA, 1996; 2004) and have been proposed as a tool to explore different internal dose metrics which correspond to different hypothesized mode(s) of action (e.g., particle number versus mass normalized by various factors such as regional surface area or number of alveolar macrophages) (Snipes et al., 1997; Jarabek et al., 2005; Brown et al., 2005). Dosimetry models for fiber deposition have previously been developed for both rats (Asgharian and Anjilvel, 1998) and humans (Asgharian and Yu, 1988), and clearance algorithms have been developed and used to extend the rat model to provide estimates of retained fiber burden (Yu et al., 1990; 1991). These same clearance algorithms can readily be incorporated into the rat and human airway architecture of the existing MPPD model to predict retained fiber burden.

The MPPD model, used for the 2005 PM NAAQS effort and in various other pharmaceutical and industry applications, is user-friendly, flexible, and has public software that offers a graphical user interface to aid computations and simulations required by toxicologists and risk assessors (http://www.ciit.org/techtransfer/tt_technologies.asp). Because it is anticipated that risk assessment calculations for LA will also need to be readily transparent and publicly available, the MPPD model has been chosen for this effort.

The Hamner Institutes for Health Sciences and the National Institute for Occupational Safety and Health (NIOSH) are currently collaborating on a project (BAS-CDC601) to update the MPPD model with algorithms to address the nanotube range (10 – 100 nm). This EPA collaborative project will update the NIOSH MPPD model effort by incorporating established deposition and clearance algorithms to extend the predictive range for fibers from 100 nm to 100 μm. This would allow the MPPD to provide predictions of deposition and retained burdens of all types of fibers from nanotubes to asbestos. The project first involves compiling the necessary algorithms to address the larger fiber range and then an implementation phase to program them into the MPPD software. Compilation of existing data for initial verification exercises will also be performed. This initial updated MPPD model can then be used immediately in simulation exercises with the available human data to evaluate different dose-response relationships. Because particle and fiber deposition has been shown to be nonlinear with respect to exposure concentration, reflecting the integration of both fiber distribution and size-dependent deposition efficiency, this first iteration of the model to predict fiber deposition is considered an important

first step to refined dose metric construction to be used in dose-response analysis (US EPA, 1996; 2004; Snipes et al., 1997; Asgharian and Anjilvel, 1998; Jarabek et al., 2005). Subsequent extension of the initial deposition model to include clearance parameters for LA will follow, based on analysis of new data from the proposed studies (*in vitro* dissolution, intratracheal instillation, and 90-day inhalation dosimetry satellite studies). Implementation of new algorithms with revised parameters will then be used to create a clearance model. These efforts will primarily be performed for the rat and human. Recent anatomical data available for the mouse (Oldham and Phalen, 2002) has resulted in the development of a dosimetry model for that species (W. Hofman, personal communication) which will soon be incorporated into the MPPD platform. Updating of this mouse model with the fiber algorithms developed for the rat and human models will be straightforward and allow quantitative interpretation of the proposed intratracheal instillation studies.

Experimental Approach:

Specific Aim 1: Create a model of fiber deposition in rats and humans.

Existing models and data will be compiled and used to develop algorithms to create a dosimetry description of fiber deposition in humans and rats. The model will describe mechanisms of fiber deposition in the entire respiratory tract regions (upper respiratory tract [URT], tracheobronchial [TB] and pulmonary [PU; all lobes]) in both species. The dimensions of the airway architecture and species-specific ventilation rates in the MPPD model will be used. The model description of deposition will entail the following critical components, based on first principles and aerodynamic properties:

- Inhalability
- Model structure for nasal breathing (rats and humans)
- Model structure for oral breathing (humans)
- Descriptions of deposition mechanisms:
    - Impaction
    - Interception
    - Sedimentation
    - Diffusion

Specific Aim 2: Implement the deposition model and upgrade the MPPD software.

Once the deposition model is developed for rats and humans, the algorithms will be implemented and model predictions verified with existing data as available. Once verified, the model will be used to upgrade the MPPD software. Simulations for specific scenarios as needed by EPA Region 8 for immediate risk assessment applications will be performed and reported.

Specific Aim 3: Extend the model to describe fiber clearance in rats and humans.

This aim will extend the deposition model for rats and humans to describe clearance mechanisms and predict retained fiber burdens. The clearance described will account for physical dissolution, fiber splitting and sequestration, mucociliary transport, and tissue

translocation. Existing data and new data from the NHEERL laboratory studies (*in vitro* dissolution, intratracheal instillation, and the 90-day inhalation study) will be used for this development.

Specific Aim 4: Implement the clearance model and upgrade the MPPD software.

Once the clearance model for rats and humans is developed, the algorithms will be implemented and model predictions verified with existing data as available. Once verified, the model will be used to upgrade the MPPD software. Simulations for specific scenarios as needed by Region 8 for immediate risk assessment applications will be performed and reported.

Specific Aim 5: Develop a model of fiber deposition and clearance in mice.

The deposition and clearance model for rats developed under Specific Aims 1 and 3 will be adjusted and used to develop a model for the mouse using recently published anatomical data and existing deposition data.

Specific Aim 6: Implement the mouse model and upgrade the MPPD software.

Once the model is developed, the algorithms will be implemented and model predictions verified with existing data as available. Once verified, the model will be used to upgrade the MPPD software.

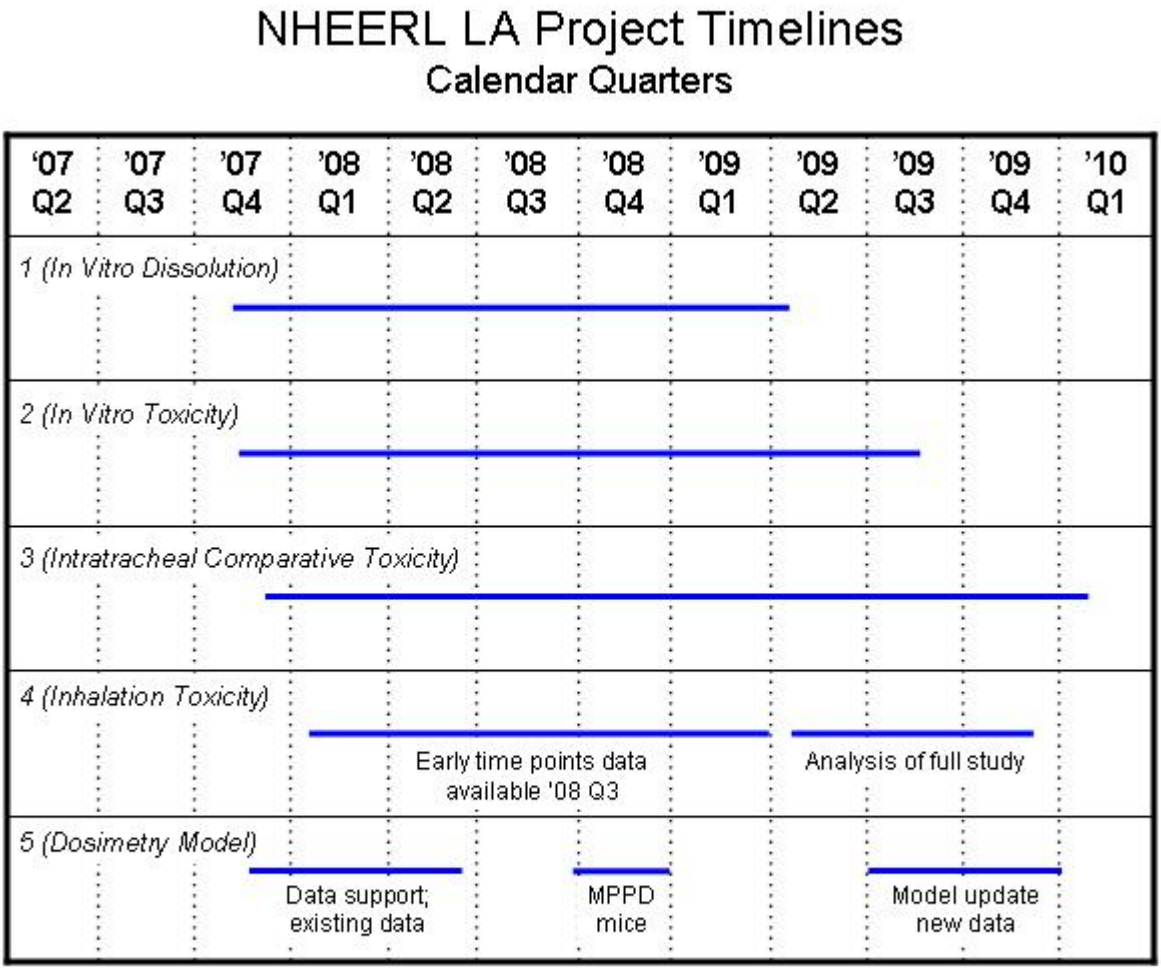
Project Resources:

Compilation of algorithms based on existing literature and data (including: inhalability, deposition efficiency, and clearance of different fiber dimensions for rat and human): PI mathematical modeler (Asgharian, The Hamner Institutes): $60,000; MPPD programmer (The Hamner Institutes): $40,000; full time equivalent (FTE; US EPA): 0.2 (Jarabek) for 6 months

Analysis and update with additional experimental data provided by *in vitro* dissolution, intratracheal instillation and inhalation dosimetry satellite studies: PI mathematical modeler (Asgharian, The Hamner Institutes) $15,000; MPPD programmer: $15,000; FTE (US EPA): 0.15 (Jarabek) for 6 months

Development and implementation of mouse architecture and appropriate adjustment of algorithms to develop MPPD version for mouse: PI Mathematical modeler (Asgharian, The Hamner Institutes): $10,000; MPPD programmer: $10,000; FTE (US EPA): 0.15 (Jarabek) for 3 months

Relative Timeline of Research Activities



Estimated Project Costs and FTE Support

	Costs	Principal Inv. FTE	Technical FTE
Overall Project Management		0.35	
Project 1 (*In Vitro* Dissolution)			
TEM sample preparation costs	$270,000		
SLF equipment and supplies	$34,000		
SEEP ($50K x 2 yr)	$100,000		
Statistical Analysis and NHEERL Database Support	$60,000		
Subtotal	**$464,000**	**0.70**	**0.50**
Project 2 (*In Vitro* Toxicity)			
Postdoc ($75K x 3 yr)	$225,000		
Student contractor ($30K x 2 yr)	$60,000		
Cell culture supplies; genomic and proteomic analysis	$110,000		
TEM sample preparation costs	$40,000		
Subtotal	**$435,000**	**0.50**	**0.60**
Project 3 (Comparative Toxicology)			
Mice and rats	$70,000		
Postdoc ($75K x 3 yr)	$225,000		
Student ($40K x 2 yr)	$80,000		
SEEP ($50K x 2 yr)	$100,000		
Gene chips	$50,000		
Lab supplies	$87,500		
TEM sample preparation costs	$200,000		
Pathology	$37,000		
NHEERL funds for 2 additional fibers	$210,000		
Subtotal	**$1,059,500**	**0.60**	**1.75**
Project 4 (Inhalation Toxicology in Rats)			
Contract subchronic nose-only inhalation exposure	$912,000		
TEM sample preparation costs	$200,000		
Subtotal	**$1,112,000**	**0.25**	**0.25**
Project 5 (Dosimetry Model)			
Existing data - Contract modeler, programmer	$100,000		
Update with new data - Contract modeler, programmer	$30,000		
Mouse MPPD program - Contract modeler, programmer	$20,000		
Subtotal	**$150,000**	**0.50**	**0.00**
Total Project Costs	**$3,220,500**	**2.90**	**3.10**

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