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

Response to Review Comments

U. S. Environmental Protection Agency
Office of Research and Development
National Health and Environmental Effects Research Laboratory
Research Triangle Park, NC

In Consultation with
U. S. EPA Region 8, Denver, CO
U.S. EPA National Center for Environmental Assessment, Washington, DC
U. S. EPA Office of Solid Waste and Emergency Response, Washington, DC

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1. Introduction

The Office of Research and Development (ORD) National Health and Environmental Effects Research Laboratory (NHEERL) conducted an internal and external review of its April 30, 2007 draft research program proposal entitled *Dosimetric and Toxicologic Assessment of Amphibole Fiber-Containing Material from Libby, Montana*, developed in consultation with EPA Region 8, ORD's National Center for Environmental Assessment (NCEA), and the Office of Solid Waste and Emergency Response. Reviewers were chosen on the basis of the need to collaborate with other Federal partners, their expertise pertaining to particular program areas, or familiarity with previous efforts on other asbestos fibers. The external peer review experts represent individuals from other Federal agencies involved with work on asbestos, academic institutions, and the private sector. Internal Federal partner reviews were provided by representatives from ATSDR and NIEHS who participated in early discussions resulting in the development of the NHEERL research program proposal. Review comments were also received from the Libby Area Technical Assistance Group, a community organization. Table 1 provides a list of the experts that provided comment. Their full original review comments are attached as appendices A through F to this document.

Table 1. Reviewers

External Peer Reviewers	**Institutions**
A. Dr. Vincent Castranova	National Institute for Occupational Safety and Health (NIOSH)
B. Dr. Brooke T. Mossman	University of Vermont
C. Dr. David B. Warheit	Dupont Haskell Laboratory
Federal Agency Reviewers	**Institutions**
D. Dr. Jill J. Dyken, Dr. John S. Wheeler	Agency for Toxic Substances and Disease Registry (ATSDR)
E. Dr. Scott Masten	National Institutes of Environmental Health Sciences (NIEHS) / National Toxicology Program (NTP)
Community Reviewer	**Institution**
F. Dr. Gerry M. Henningsen	Libby Area Technical Assistance Group (LATAG)

The purpose of this document is to provide a compilation of review comments and their disposition. Comments have been categorized into the following areas: general comments on the research program (Section 2), test sample selection and preparation (Section 3), dosimetry model development and simulation studies (Section 4), *in vitro* dissolution (Section 5), *in vitro* toxicity (Section 6), comparative toxicity in mice and rats (Section 7), and inhalation toxicology in rats (Section 8). These categories correspond to the major projects in the draft research program proposal. The goal of each project is provided again herein to provide an introductory background on the objectives of each project. The reader is referred to the Peer Reviewed Revised Draft document available separately, for additional detail on experimental approaches.

Major topics within each comment category are summarized, with the individual comments provided in each section. NHEERL responses for each topic are indicated following each topic. Comments on the proposal section entitled *Comparisons to the Existing NHEERL*

Mineral and Synthetic Fiber Dose Characterization Database have been incorporated back into Section 5 on the *in vitro* dissolution studies for proper placement and context according to the revised NHEERL plan. Section 9 provides additional references cited in these responses to peer comments.

In general, the reviewers (with one significant exception) were very supportive of the type of studies described in our research approach. We have addressed all significant comments by the reviewers in this document, and we have explained how the comments have affected our planning for these studies. We are working diligently to resolve all issues necessary to start the work. Funds have been allocated for these projects, and students, postdoctoral fellows, and contractors are being recruited to help us with this work. NHEERL and its partners in EPA Region 8, NCEA, and OSWER are eager to address the critical health effects issues of LA and other samples of naturally occurring asbestos.

2. General Comments

2.1. Scope and Utility of Proposed Program.

The reviewers generally agreed that the proposed research was comprehensive and appropriately addressed critical needs regarding health assessment of asbestos-related disease. Further, it was recognized that asbestos-related disease was an important environmental problem of significant population impact. We are very pleased that all but one reviewer agreed that the scope and utility of the proposed projects is appropriate to address these critical needs. One reviewer (LATAG) disagreed with the scope and proposed program (see Section 2.5).

- *(ATSDR (Dr. Dyken)): This is an ambitious and thorough research plan which, if implemented fully, would add considerably to the body of knowledge of how Libby amphibole and other asbestos-related minerals contribute to disease.*

- *(ATSDR (Dr. Dyken)): The proposed research projects were evaluated in light of the following five principles for setting public health research priorities:*

1. *The research project itself protects public health. (e.g., a study of exposure mitigation strategies).*
2. *The research project provides information essential for making evidence-based cleanup or exposure mitigation decisions (e.g., dose–disease response analysis to set appropriate cleanup levels).*
3. *The research project assists governmental agencies and partners in making appropriate policies affecting public health (e.g., research showing regulation is needed to prevent hazardous exposures).*
4. *The research project serves community needs and concerns (e.g., studies of disease diagnosis and treatment).*
5. *The research project advances the basic science upon which public health decisions are made (e.g., basic science leading to understanding of disease mechanisms).*
    - *NHEERL's proposed research projects generally meet principle 5 in advancing the basic science describing how Libby amphibole materials cause disease and how the toxicity relates to other (better studied) asbestos materials.*

- *(Dr. Warheit): The proposed studies represent a multidisciplined attempt to address the key areas of uncertainty related to Libby asbestos health risk assessments.*

- *(Dr. Warheit): The proposed in vivo studies appropriately address the hazards of Libby amphibole asbestos fibers.*

- *(Dr. Mossman): This document describes proposed research over a 3 year period of funding to address the toxicology in vitro and in vivo of amphibole fiber-containing vermiculite ore from Libby, Montana that has resulted in an excess of both malignant and nonmalignant respiratory disease in workers and citizens of this community. In addition, the product was sent to other plants in the US for processing and is thought to exist in at least 30 million homes. Thus, the research proposed is highly significant as little is known about the toxicology of the Libby ore, its products, and risks to exposed populations of workers and citizens residing in homes using these products. Because of the mineralogical uniqueness and complexity of the Libby amphibole (LA), it does not fall under the regulatory sphere of the six types of 'asbestos' fibers as defined by the EPA and other government agencies. For these reasons, the studies proposed are also mandatory to determine how the biological effects and solubility of the Libby ore/products compare to the classical asbestos types.*

- *(Dr. Mossman): Yes the research projects produce data and products useful to EPA Region 8 which must provide technical support to the Libby, MT community. Hopefully, data will reveal whether or not the risk of exposure to LA reflects the same health risks and diseases seen with different types of asbestos and/or explain the unique pleural fibrosis that is seen in the Libby population that has not been observed thusfar in animal models of asbestos exposure. Moreover, studies should elucidate the components of LA that are pathogenic. Lastly, they will determine whether certain populations (the young, the elderly, compromised individuals, etc.) are more prone to LA-associated diseases.*

- *(Dr. Mossman): Yes, the proposed in vitro, in vitro and dosimeter studies address the risk assessment of Libby appropriately. The research plans are developed to characterize key features of the Libby Amphibole (LA) that will predict its internal dose/retention. Proposed research will characterize molecular and cellular mechanisms of toxicity and tissue reactions leading to disease endpoints in rodents. In addition, they will predict the inherent toxicity of LA in comparison to asbestos types and whether there is differential sensitivity to LA or asbestos types related to age or other pathologies. The use of well-characterized and sized positive (amphibole asbestos) and negative (nonpathogenic fibers or particles) controls is imperative as well as determining the most appropriate basis for equivalent comparisons between particulates (surface area, fiber number, fiber size) prior to the initiation of long-range or animal studies.*

- *(Dr. Mossman): In summary, the proposed studies have many strengths and the credentials of the investigators listed are outstanding. Hopefully the EPA or other agencies are also initiating epidemiologic and clinical studies on the Libby population to elucidate biomarkers, enable therapeutic strategies and find risk factors in humans now afflicted with LA diseases, both malignant and nonmalignant.*

- *(Dr. Masten): The project outline is well-written and thorough. Most of our comments focus on the relative utility and priority of the described projects, particularly as related to ensuring that the NTP can make use of the resulting analytical methods and toxicity data in developing a research program to characterize chronic toxicity/carcinogenicity of Libby amphibole (LA) and related mineral fibers in long-term inhalation studies.*

2.2. Ability to Complete All Projects in Required Time Frame.

Some reviewers questioned whether the scope of the studies could be completed in the required time frame.

- *ATSDR (Dr. Dyken)): However, because of significant time and resource commitments associated with the proposed plan, it is not clear that conclusive results will be available within a time frame that would allow important risk management decisions to be made for the Libby community.*

- *(Dr. Mossman): Although the studies seem overambitious for a 3 year period, they address all of the key mechanisms of action that have been reported for asbestos including tests for durability (in vitro dissolution data, tissue/fiber burden studies in animals), epigenetic effects (cell signaling, cytokine production, proliferation) and genetic (chromosomal and other genetic abnormalities) effects. Subchronic inhalation studies in rodents will complement these mechanistic studies and document disease potential.*

- *(Dr. Castranova): The studies are for too ambitious. Both the ILSI Workgroup to develop a short-term testing strategy for fibers sponsored by EPA (Bernstein et al., 2005) and the EPA Panel for chronic fiber testing (Test Guidelines for Chronic Inhalation Toxicity and Carcinogenicity of Fibrous Particles, Sept. 2000) stated that the rat was the preferred species for testing. In general, mice are less sensitive to fibrosis. I suggest limiting IT studies to rats.*

NHEERL Response: We recognize that due to typical research barriers and pitfalls certain studies may not be completed in the required time frame. For this reason, the revised version contains further details on the overall priorities of the listed projects, including which projects are critical for adequate toxicity comparisons and assistance in risk assessment activities.

2.3. Timing and Integration of Projects.

Dr. Warheit was concerned about the timing and integration of the projects within the proposed program.

- *(Dr. Warheit): Although this Reviewer was not present at the January, 2007 planning meeting in Research Triangle Park to discuss research priorities, I have only moderate enthusiasm for the proposed plan of action to conduct dosimetric and toxicological assessments of the Libby amphibole material. In this regard, the strengths of the plan of action are associated with the planned intratracheal comparative studies, followed by the 90-day inhalation studies. The weaknesses of the project are associated with the planned in vitro toxicity studies. In addition, it is unclear whether the in vitro dissolution studies will be instructive; and finally the dosimetry model, if developed properly, could be very useful but should only be applied following the generation of in vivo (intratracheal and/or inhalation) data.*

- *(Dr. Warheit): The proposed in vivo studies appropriately address the hazards of Libby amphibole asbestos fibers. The utility of the proposed dosimetry studies would be enhanced contingent upon first obtaining reliable in vivo lung deposition, clearance and pathology data. The in vitro toxicity data could be utilized to test mechanistic hypotheses generated from the*

results of the in vivo studies. The in vitro dissolution studies should be carried out in conjunction with in vivo biopersistence.

NHEERL Response: We agree that the dosimetry studies would be enhanced by first obtaining the *in vivo* data, and that the that the *in vitro* dissolution studies should be carried out in conjunction with *in vivo* biopersistence studies, The reviewer's statements reflect what we have already proposed. The initial dosimetry model can be constructed without *in vivo* data, but new data from the NHEERL *in vivo* studies will be used to extend and refine the model. To emphasize this point, the dosimetry section project has been moved to the final set of studies in the revised NHEERL plan, and the sequence of studies to support its development has been clarified. We are planning to conduct *in vitro* dissolution studies in conjunction with the *in vivo* biopersistence (fiber burden) studies.

Dr. Warheit recommended that due to "little correlation" between relative *in vitro* and *in vivo* toxicity results, the *in vitro* studies should be conducted after completion of the *in vivo* studies, and used to test mechanistic hypotheses generated from the results of the *in vivo* studies.

- *(Dr. Warheit): The current research plan needs to be revised according to the strategies outlined in other comments. The timelines for the various research projects should be reorganized as described. It is strongly suggested that the in vivo studies precede the development of in vitro dissolution, in vitro toxicity and dosimetry studies. This may not be the most convenient strategy but clearly will lead to the most productive and accurate results. There is no doubt that successful completion of the intratracheal and inhalation studies could provide very useful data for assessing the hazards of Libby amphibole fibers in the lungs of rats. The in vitro toxicity studies could provide useful, hypothesis-driven, mechanistic data, but should not be utilized for preliminary screening evaluations or hazard assessments.*

- *(Dr. Warheit): With respect to the proposed in vitro toxicity data, previous studies have reported little correlation between the relative toxicity of particles when comparing lung toxicity rankings following in vivo instillation compared to in vitro cell culture exposures (Seagrave et al., 2002; 2003; 2005). Moreover, a recently published study was designed to assess the capacity of in vitro screening studies to predict in vivo pulmonary toxicity of several fine or nanoscale particles in rats. The authors concluded that in vitro cellular systems will need to be further developed, standardized, and validated (relative to in vivo effects) in order to provide useful screening data on the relative toxicity of inhaled particles (Sayes et al., 2007). Moreover, the ILSI Risk Science Institute Working Group on testing assays for fibrous particles concluded that current in vitro tests systems have limited usefulness for hazard identification or characterization of dose response relationships (ILSI – Bernstein et al., 2005). In addition, it was suggested that for some fiber-types, these tests may help to identify and evaluate possible mechanisms involved in fiber-related lung pathogenesis. But this concept was viewed as supplementary to the identification of fiber-induced toxic effects by utilizing in vivo methodologies. Thus, it is suggested that any in vitro toxicity studies designed to assess hazard potential of these amphibole-like materials should only be conducted after completion of in vivo tests and the predictability of in vitro tests can only be validated using in vivo results.*

Response: We disagree with this viewpoint. *In vitro* studies can serve as a foundation for *in vivo* studies by examining the relative potency and mechanisms of action of a large number of samples. The information from these studies can give highly relevant information for the selection of samples and conduct of the *in vivo* studies. In this respect, *in vitro* studies serve as a

base for a "pyramid" of studies, with *in vitro* studies informing instillation studies, which inform inhalation studies at the top of the pyramid, which due to resource limitations can only be conducted on a very limited set of samples. We and others have published studies in which results obtained from *in vitro* toxicology experiments were shown to be predictive of *in vivo* responses. NHEERL's PM program has a large and successful component in which *in vitro* toxicology is used to screen samples of different size, chemistry, and geographical origin, and a subset of samples used for animal studies. Regional EPA representatives have been very enthusiastic about the use of *in vitro* experiments as a preliminary screen. We recognize that *in vitro* studies have limitations, but the approach recommended above is contrary to that which we have found to be quite successful. Please see the responses to comments on the *in vitro* studies for further explanation of our *in vitro* approach.

2.4. Overall Priority of Projects.

Reviewers had different overall priorities for the conduct of the individual projects studies in the NHEERL plan.

- *(Dr. Masten): In summary, we view the materials characterization, dosimetry model development and thorough subchronic inhalation studies as the highest priority projects that will have the most impact on the design of NTP studies as well addressing current asbestos risk assessment uncertainties. We look forward to collaborating on implementation of this research program and offer assistance in specific study protocol development.*

- *(Dr. Dyken): Model development to describe internal fiber burden. As detailed above, this project would be useful for long-term research but unlikely to contribute significantly to upcoming risk management and policy decisions at Libby.*
  - *In vitro dissolution studies. These studies will be useful for comparative purposes and would supply information needed for model development. However, it is already known that amphiboles in general dissolve much more slowly and thus are much more biopersistent than, for example, chrysotile asbestos. It is questionable whether new findings on dissolution rates specific to Libby amphibole would significantly alter cleanup decisions.*
  - *Animal intratracheal injection studies. These studies would also be useful for comparative purposes with commercial asbestos materials and, in combination with current scientific knowledge, might allow determination of correction factors that could be used to apply risk models developed for commercial asbestos to Libby amphibole. To use the results directly in setting cleanup goals, however, findings on the basis of intratracheal dose would have to be correlated with and/or translated to an air concentration.*
  - *Animal inhalation studies. These studies could provide data that would directly assist in setting cleanup levels, since exposure would be based on air levels.*
  - *Translocation studies. These studies may be useful to answer basic research questions, although researchers have already shown translocation of amphiboles. It is questionable whether new findings on translocation specific to Libby amphibole would significantly alter cleanup decisions.*
  - *Studies on more-sensitive disease outcomes. These studies may be useful for basic health intervention research. However, it is questionable whether findings would practically influence cleanup levels at Libby. Cleanup levels selected based on pulmonary endpoint-based risk may likely be near the limit of economic feasibility.*

NHEERL Response: We believe that all of our projects are equally important in addressing the risk assessment uncertainties. We agree with Dr. Dyken that further exposure assessment is necessary, and these needs are addressed in current investigations by the Region 8 Libby team. The team is investigating three important pathways of exposure – outdoor ambient air, outdoor activity-based exposures, and indoor activity-based exposures. Other exposure pathways have been identified and will be addressed as warranted.

2.5. "Basic Research" vs. "Useful Applied Data" and Research Planning Process.

The LATAG and their Technical Advisor, Dr. Henningsen, were quite strong in their opinion that that the NHEERL research represented basic research and questioned its utility for risk assessment application by Region 8. A new process was proposed to arrive at a research program. Key sentences are quoted here; please see Appendix D (p. 1-2) for the full context

- *(LATAG): I conclude (while acknowledging excellent scientific capabilities and reputation of NHEERL) that the proposed research projects are misdirected. NHEERL missed the main purposes of supporting R8 for conducting the most urgent and practical toxicological studies of LA, that are critically needed to supply essential site-specific data for quantitative risk assessment of Libby. Current lab proposals will certainly fall short of the goals and needs of R8 and Libby to obtain usable data (derived from applied research studies and prioritized site-specific investigations), which are urgently required. … In other words, R8 and Libby do not need so much basic research that is currently proposed by NHEERL, but R8 mostly needs realistic "testing" of the toxicity on (sic) LA asbestos for prioritized risk-based data gaps; e.g., determine the "relative toxic potency" of representative LA asbestos fibers, compared to standardized samples of chrysotile and amphibole fibers. While the mostly basic research that is outlined in the current draft proposal may be interesting, it has little practical use for helping NPL sites that are contaminated with amphibole fibers. … I suggest that EPA should essentially start this research project anew, and adopt a practical site-specific approach for proposing applied lab research and risk-based site investigations of LA asbestos at Libby. EPA should create a small, effective panel of outside expert scientists who can independently and objectively review EPA's proposed research … This panel should be tasked to objectively apply best available science to help EPA clearly define or refine risk management goals, prioritize study objectives, review study proposals/results/reports, and communicate risks of LA asbestos, with focus on site investigations that fill critical data-gaps and reduce uncertainties of risks. For equitable scientific representation of Libby, the LATAG should name at least 6 expert scientist members to this panel.*

NHEERL Response: It is very important to understand that the NHEERL plans described here are only designed to address issues related to toxicity and dosimetry of Libby amphibole (LA), and two samples of naturally occurring asbestos. The five projects described in the NHEERL plans are only part of the Libby Action Plan approved by the Office of Solid Waste and Emergency Response (OSWER) in February 2007. This plan also includes work by Region 8, ORD/NCEA, OSWER, and the USGS to address the issues of sample preparation and characterization, fiber size distribution, LA RfC development, LA cancer assessment, new epidemiological information from the Libby Montana cohort and other cohorts, and the OSWER interim risk methodology for quantification of risk assessment from inhalation exposure to asbestos. The entire budget for these activities is currently about $6.8 million (the NHEERL portion is $3.3 million), and it is likely that more funds will be available in future years to

supplement these studies. The high-priority studies listed by Dr. Henningsen as necessary for remedial cleanup and protection of citizens are on the whole covered by the entire Libby Action Plan.

We also disagree strongly with the sentiment that the proposed NHEERL studies are "basic research" unlikely to be useful for risk assessment. These studies have been developed after careful and extensive consultations with Region 8 and NCEA. NHEERL will be testing the *in vitro* dissolution, *in vitro* toxicity, and *in vivo* toxicity, and inhalation toxicity of LA fibers in comparison to other fiber samples. This data will be used to adjust an *in vivo* dosimetry model for LA fibers. We believe that these activities will contribute important data for the risk assessment of LA. In the course of these studies, many endpoints will be measured to assess relative toxicity, both *in vitro* and *in vivo*. Some of these endpoints may not have direct quantitative applicability to risk assessment, but they can inform mechanism and mode of action which will help us to understand the unique toxicity of LA relative to other asbestos fibers. Additionally, data from some of the NHEERL studies will be integrated into other sections of the Libby Action Plan, such as the use of biomarkers in prospective epidemiological and clinical investigations.

The perceived "misdirection" of the NHEERL research plan is not an accurate assessment of the role of NHEERL in the overall Libby Action Plan. We do agree that the research strategy, study objectives, relative priorities, and how the research data will be used in informing the Record of Decision can be more clearly defined. To that end, we have made several changes throughout the document to clarify these points. We do not agree that an outside expert panel could start the process over and achieve more effective results. In fact, the external reviewers chosen are all experts in relevant areas. This option would result in delays of many months during which time experts are recruited, meetings conducted, strategies devised, and final consensus is reached. Another extremely important consideration is that NHEERL and Region 8 personnel understand their own resources and capabilities, while an outside panel may recommend studies which may not reflect realistic possibilities. The end result of an outside expert panel is certain delay, no guarantee of a better plan, and the possibility of a worse plan due to limited understanding of current resources and expertise. We encourage ongoing communication with LATAG so that they may gain confidence in the research plan we have described.

2.6. Application to "Record of Decision"

One reviewer was concerned with how the analysis of resultant data would be applied in a "Record of Decision" by Region 8 at the Libby site.

- *(Dr. Mossman): While the compendium of proposed toxicologic studies is robust and likely to provide mechanistic information and disease in rodents, it is also unclear how analysis of all data will be amalgamated and incorporated into a "Record of Decision".*

NHEERL Response: Results of the NHEERL program will be communicated regularly to Region 8 risk assessors so that actions at the site can reflect the most recent science. It is anticipated that the data from the program as well as inferences and applications will undergo typical Agency review and scrutiny so that they can be utilized in decision making.

3. Test Sample Selection and Preparation

A number of reviewers expressed the view that characterization and preparation of the test sample of LA to be used in the research program was of critical importance. These comments are captured in this section.

3.1. Characterization of Test Samples Is a Critical Step.

Several reviewers identified proper characterization of the LA sample and other test materials as a critical step in order to proceed with initiation of the program projects.

- *(LATAG): This is the most critical step to conduct accurately for all comparative samples PRIOR to starting any research studies.*

- *(Dr. Masten): The use of different test materials for the in vitro and in vivo studies (i.e. 2000 vs 2007 sample collections) is a significant risk that should be carefully considered. At a minimum, the new sample should be first collected, characterized and compared to the old sample. … How does the test material to be used compare to Libby environmental sampling? We recommend focusing more effort on demonstrating the relevance of materials that are prepared and selected for study to human exposure situations. This is work that EPA is well suited to do and is crucial for dosimetry model development and toxicity data interpretation. … Test materials should also be characterized for fiber type, size etc. after fractionation.*

- *(Dr. Castranova): There is a rush to conduct toxicology (in vitro dissolution, in vitro toxicity, and IT and inhalation studies are scheduled to start by Y 07 Q4). Due to the urgency to begin studies, little attention is given to sample preparation, which is critical to program success. … There is danger in using 2 Libby samples collected and prepared at different times (in 2000 vs 2007). It is unlikely that the two samples would be identical in fiber count/mg; types of contaminating particles, fiber dimension, etc. It would be preferable to prepare and well characterize a single Libby sample to be used for all projects throughout the program duration.*

NHEERL Response: EPA absolutely agrees that careful sample preparation and characterization are critical steps which must be carried out on all asbestos samples. This characterization, for all samples tested, is emphasized in the research proposal. We do plan to initiate the *in vitro* and instillation toxicology studies with the 2000 LA sample. When the 2007 LA sample is available, we will conduct side-by-side comparisons of the two in the *in vitro* and instillation toxicology studies. The 2007 sample will be used for the subchronic inhalation study and also will be used by NTP for their 2-year chronic study.

3.2. Clarify Test Samples for Different Projects.

Reviewers wanted clarification on the test sample to be used in different projects.

- *(Dr. Masten): We realize that the identification and selection of test materials is difficult and unresolved but insufficient detail is provided to enable external reviewers to understand the scope of work and interrelatedness of research projects. What studies will be conducted on what*

materials? … Comparisons to commercial asbestos (i.e. amosite) are important but the value of including SVFs, wollastonite as comparison is not clear.

- *(Dr. Castranova): Wollastonite would be a more appropriate negative control fiber, since glass fibers are likely to be thicker than amphibole fibers.*

NHEERL Response: In the revised document, we have specified more clearly the samples to be tested. We agree that SVFs and glass wool are not as useful to test as the other asbestos samples, although they may be useful in considering the development of the dosimetric model**.**

3.3. Choice of Positive Control.

Dr. Castranova recommended the use of tremolite asbestos as a positive control for the toxicology studies.

- *(Dr. Castranova): The Libby and El Dorado amphibole exposures are most like tremolite in chemical nature. Therefore, chrysotile and refractory ceramic fibers would be poor positive controls. Amosite would be satisfactory, but fibrous tremolite would be the most relevant positive control.*

NHEERL Response: Although it is true that the Libby and El Dorado samples may be more like tremolite than amosite, the main purpose of the control is to obtain a positive inflammatory, pathological, and fibrotic response. For this reason, amosite remains the better choice considering the extensive toxicological database on this fiber type.

3.4. A Recommended Approach to LA Sample Preparation.

Two reviewers offered specific comments on how to prepare and characterize test samples for use in the studies.

- *(Dr. Mossman): Although the primary focus of all studies is on the LA material (presumably the Six Mix from 6 different sites in Libby) characterized in 2000, comparing this complex mix of fibers, fragments and particles to asbestos fiber samples or other non-asbestos amphiboles may reveal little effects (as several laboratories have already reported) because of the small fiber:particle ratio. Moreover, testing may not reflect the toxicity of the product (Zonolite) used in homes. Sizing or fractionation of the LA and final product for enriched fiber:particle or elongated fiber:particle preparations would be preferable to examination of site-specific chrysotiles or taconite-associated amphiboles.*

- *(Dr. Castranova): I suggest that a major effort be placed in preparation and characterization of fiber samples.*

  a) Prepare a single Libby sample to be used in all studies. Characterize the fiber count/mass, the fiber length distribution, the amount of non-fibrous particles/mass, and the composition of non-fibrous particles.

  b) The design states that effort will be made to remove non-fibrous particles with an aerodynamic diameter of >10 µm. Effort should be made to remove as much non-fibrous material of all sizes as possible to enrich the Libby amphibole in the sample. This may prove very difficult. An alternative is to use tremolite fibers as the positive control. Mill the fibers

to shorten the length to be comparable to the Libby fibers. Lastly, dope the tremolite sample with non-fibrous particles of similar composition and amount as the Libby sample. In these ways, the Libby sample and the positive control would be more comparable.

c) The negative control fiber sample should be milled and doped in a similar manner.

d) An additional particle control could be a sample of non-fibrous particles of similar size and composition to those found in the Libby sample.

e) Wollastonite would be a more appropriate negative control fiber, since glass fibers are likely to be thicker than amphibole fibers.

f) Berman and Crump conducted a meta analysis of epidemiological data with asbestos and lung cancer for EPA (Technical support document for a protocol to assess asbestos – related risk, 2001). In this analysis, they found that fibers > 10 µm in length were substantially more carcinogenic than those from 5 – 10 µm. This points to the importance of having similar fiber lengths in the Libby and the positive control samples.

NHEERL Response: We shall consider attempting to fractionate the LA material and testing whether certain components of LA are more toxic. Besides the very significant technical hurdles to overcome with the approaches suggested here by the reviewers, we must consider the fact that people are exposed to the whole LA material as a mixture, and it will be most relevant to test samples of LA which are representative of this mixture. Therefore it seems the greatest benefit will be to characterize the properties and toxicity of the respirable fraction of the original LA material. If we can find a reasonably effective technique to enrich the LA and NOA samples for longer fibers, then we will use these enriched samples in some of our comparative *in vitro* and *in vivo* instillation toxicity studies. However, it is very unlikely that enough enriched sample could be collected to conduct the subchronic inhalation study or NTP chronic inhalation study

4. Dosimetry Model Development and Simulation Studies

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.

The range of peer review comments on this project was large, from suggestions to using the model to inform experimental design to prioritizing the project as low. While support was expressed generally, for example by Dr. Masten, some reviewers had concerns with respect to uncertainty in the input data and utility of risk estimates. Others were only concerned about timing of its implementation so that it was based on the experimental data. Finally, application of these models versus current empirical model exercises was also a concern. These are summarized in the following sections.

4.1. Prioritization and Utility

Reviewers from the ATSDR and the LATAG expressed concern that the uncertainties in exposure measurements for the epidemiological studies would prevent the dosimetry modeling effort, aimed at describing internal fiber burdens in both humans and rats, from contributing to cleanup-related decisions. The timeliness of the project was also questioned, suggesting that it was of more benefit to “long-term research”.

- *(ATSDR (Dr. Dyken)): I recommend that NHEERL prioritize toxicological research based on fiber air concentrations over research based on modeled internal fiber burden. I recognize that internal fiber burden may be the more toxicologically relevant dose metric. However, given the current state of science, it does not contribute in a practical way to cleanup-related decision making for the following reasons:*
  - *Epidemiological studies upon which asbestos risk assessments depend describe only (highly uncertain) air fiber concentration measurements;*
  - *The relation of an animal dose to realistic human exposure dose or fiber burden is unknown and unlikely to be available in a reasonable period of time: and*
  - *The correlation between human fiber burden and adverse health outcome is unclear and unlikely to be determined in a reasonable period of time.*

NHEERL Response: To first clarify, the NHEERL program does not prioritize either the toxicological research based on fiber air concentration or the modeling exercises to simulate internal body burdens. Rather than one over the other, the proposal is for an integrated approach that iteratively develops the dosimetry model and provides for hypothesis testing (e.g., does a certain fiber size correlate with observed lesions in a given location) and dose-response analyses to aid inferences drawn from the results of the toxicological studies. Further, reliance on external exposure, as recognized in the comment, is not as relevant as internal dose for predicting toxicity. This is the basic tenet of contemporary molecular epidemiology and the increase in accuracy afforded by computing internal dose for response analysis is recommended for improvement of risk assessment in both Agency guidance and by the NRC (U.S. EPA, 1994; 2005; NRC, 1991; 2007).

The uncertainty in the fiber air measurements should not be used to preclude mechanistic modeling. In fact, issues of differences in fiber distribution and potency can be formally and explicitly explored with the more mechanistic dosimetry models. Specific questions with respect to fiber distribution, durability, and biopersistence can be explored quantitatively with the mechanistic model as it readily supports the simulation of different exposure scenarios and the calculation of different dose metrics. It should be noted that an important recommendation for further study regarding the proposed protocol to assess asbestos cancer risk was to "define adjustments for potency factors that will allow them to be used with an exposure index that even more closely captures asbestos characteristics that determine biological activity" (Section 8.3, Page 8.13). The "Kl" and "Km" terms in that model represent the slope factors derived from a stratification of the response measure (i.e., lung cancer versus mesothelioma incidence). The proposed NHEERL dosimetry model addresses this recommendation with species- and fiber-specific mechanisms of inhalability, deposition, and clearance, support by LA-specific parameters. Thus, the proposed project has attributes which specifically address the deficiencies of the existing empirical models and may provide both comparative potency as well as its underlying determinants. Further, with respect to interspecies extrapolation, existing Agency methods rely on mechanistic models as the preferred approach and fiber-specific models have already been published (U.S. EPA, 1994; Jarabek, 2005; Moolgavkar et al., 2001).

- *(ATSDR (Dr. Wheeler)): A large portion of the proposed research relies on developing a model to describe internal fiber burden. While this could be useful for long term research, it is unlikely to contribute knowledge that will be timely in making risk management decisions for the Libby site for the above reasons. Several of the proposed additional research projects in turn rely on*

the (to-be-developed) model for translating the findings to information that would be practically useful for guiding risk management and policy decisions. These projects, while valuable for long-term research, may not be helpful for the short-term decision making needs at Libby.

NHEERL Response: As described above, the dosimetry model project is only one of five in the overall program. Performance of the toxicological testing projects proposed in the program does not rely on the modeling project, but rather both the computational and experimental components will be pursued in an integrated and iterative fashion. The fiber inhalability and deposition efficiency algorithms, based on first principles and aerodynamic properties of fiber length and diameter, have already been peer reviewed and published so that the initial human model can be exercised to arrive at insights relevant to the Libby site within the first year.

- *(ATSDR (Dr. Wheeler)): Obtaining the correct dose metric for evaluating toxicity from asbestos exposure has been an ongoing effort in the asbestos field for several decades. Developing a method to calculate internal dose of the biologically active forms of asbestos fibers (mineralogical and morphological) should enable the health/risk assessment field to better predict disease. It will also allow the collection of data that will help elucidate asbestos' mode and mechanism of action. The development will, however, require the examination of many model variables, will require a considerable research effort, and probably will not be timely enough to help in making clean-up decisions in Libby, Montana. Some concerns are listed below:*
  - *Trying to model the epidemiology studies has severe limitations. Not only is there wide uncertainty about the exposure estimates but there is little information available about the fiber size dimensions. Internal distribution estimates would be meaningless without the proper fiber distribution data. The EPA's main concern with the Berman-Crump methodology was the surrogate data they employed to obtain fiber size distributions for the epidemiology studies. This proposal does not improve that limitation and is thus subject to the same criticism.*
  - *While there is evidence that longer fibers may increase toxicity, the role of fiber dimension is still undergoing considerable debate, especially for non-cancer endpoints. For developing a model, it could be assumed that fibers deposited in the bronchiole tree would lead to bronchogenic carcinomas and that fibers deposited in the alveolar region could lead to interstitial fibrosis. However, the understanding of deposition leading to mesothelioma is much less certain. While it appears that long fibers lodge in the lung and are therefore available to move to the mesothelium, microscopic examination of mesothelioma tissue shows short fibers. To clarify this picture for use in a deposition/clearance model, a major research effort would be required.*
  - *Clearance mechanisms are complex in asbestos analysis. A major hurdle would be that clearance would be dependent upon fiber size distributions that are unknown. In the case of lung cancer and asbestosis, clearance from the lung is likely beneficial and leads to decreased toxicity. Clearance in the case of mesothelioma and pleural disease could lead to less toxicity through macrophage clearance but to greater toxicity if cleared into the pleura. Even if the dose-response of this movement of fibers into the pleura was unknown or understood, the size fibers being cleared into the pleura that lead to disease is unknown.*

NHEERL Response: As above, the deficiencies of the epidemiological data should not preclude development of mechanistic models that may in fact facilitate inferences regarding determinants of comparative potency and relevant dose metrics. And to be clear, dosimetry models do not introduce any more uncertainty than are already in the exposure-response

functions, and likely will result in greater accuracy. Surrogate data will not need to be used in the mechanistic model as any fiber distribution can be simulated and in fact, bounding exercises would inform discussions of any potential uncertainty. Further, a significant portion of the program, as described in Section 3 above, will be devoted to analysis and characterization of the Libby site sample that will be used in the studies.

The model development and simulations exercises for dose-response analyses need not *a priori* make any assumption regarding plausible dose metrics such as those provided in the comments. Indeed, the flexibility and versatility to systematically and quantitatively explore various hypotheses regarding plausible mode(s) of action by virtue of ready calculation of dose-response analyses using different dose metrics, afforded only by a more mechanistic model structure, is one of the most compelling motivations for its development. Concerns for unknown fiber size distributions can actually be explored by explicit simulation of different distributions with different variances. Correlation of the different dose metrics can be calculated based on the fiber burdens measured in various tissues from the experimental studies. Other concerns enumerated here are all essentially with respect to potential response dynamics. Simulations with different dose metrics may readily reveal insights that inform key determinants of the response dynamics (e.g., fiber number versus mass is more explanatory).

- *(ATSDR (JW)): At this time it appears that a better use of limited research funds would be to perform studies that accurately predict fiber size distributions in historical epidemiological studies. These values could be used both in the OSWER Interim Guidance and any future internal dose calculations.*

NHEERL Response: Simulation studies of internal dose can be used to back extrapolate and evaluate the degree of uncertainty that would depend on different exposure distributions so that we would instead argue its development should precede any retrospective analysis of exposure data. For example, if the exposure did not characterize a certain size fraction well that is also not deposited efficiently in the respiratory tract, then that error in exposure measurement may be less important. Also, current plans include better characterization of LA dimensions.

4.2. Timing and Integration

Those reviewers who favored the model development were primarily concerned that its implementation be timed to be based on the experimental data obtained in the other projects. Dr. Masten from the NTP also suggested that the modeling be used to inform the experimental design.

- *(Dr. Warheit): The dosimetry (deposition/clearance) data should not precede the in vivo data, but rather should be implemented after the generation of in vivo data (preferably morphometric data – which is not planned in this project) has been successfully achieved.*

NHEERL Response: We agree with this statement, which reflects our original intent. As described in Section 2 of this Response, the dosimetry project description in the revised program proposal has been moved to be the last section to emphasize this integration, and the text in the proposal has been revised to reinforce that the only aspect of the dosimetry model that will precede in parallel with the initiation of the experimental studies is the coding of the existing

algorithms for fiber inhalability and deposition efficiency, based on first principles and aerodynamic properties, into the MPPD menu. The LA-specific bivariate distributions (i.e., fiber distributions characterized by length and diameter) determined in the tissues of the *in vivo* projects will then be used to refine and derive LA specific parameters such as clearance rate constants.

- *(Dr. Warheit): The dosimetry models can provide important information, but the success of this project is dependent upon having reliable in vivo data. Lung morphometry data on (inhaled) amphibole fiber deposition, clearance, and pathological progression-type data would be the most useful source for developing dosimetric models. Alternatively, in vivo lung morphology assessments would be adequate. However, it is unclear how progress on dosimetric assessments could be advanced in the absence of generating data associated with in vivo lung tissue evaluations.*

NHEERL Response: We agree with this statement. For this reason, the proposed experimental design includes serial sacrifice and fiber burden determination in various key tissues to be evaluated in both the intratracheal and inhalation projects.

- *(Dr. Masten): Is there an opportunity to use the existing or updated MPPD model to aid in designing/setting doses for the inhalation studies?*

NHEERL Response: Yes, the published algorithms for fiber deposition and clearance on which the update in code and extension of the MPPD model will be based can provide insights for the design and setting of doses for the inhalation project.

4.3 Applications and Inferences

Some divergent concerns regarding application of the dosimetry model and reliance on it for risk inference were expressed by LATAG.

- *(LATAG): The models can be used to estimate various endpoints of disease, but they must be validated for their predictability and calibrated for use at specific sites with unique conditions. Therefore, modeling of risks from estimated dosimetry inputs and kinetic assumptions is highly uncertain and should be relegated with lower priority to later times.*
- *(LATAG): Main categories of site studies that are needed with high priority include: Computational support to organize data into usable forms and to model (if valid for fibers and calibrated) exposure and dose response estimates that include estimates of variability and uncertainty.*
- *(LATAG; GH75 comment): What about comparative models, such as Bermann-Krump (sic) – why not compare their results?*
- *(LATAG; GH79 comment): No mice! [refers to comment in letter that "mice are not needed"].*

NHEERL Response: These comments by the LATAG appear to be directly divergent. Despite calling for computational support to organize and model data that will include estimates of variability and uncertainty, the project that has been proposed to provide that capability is suggested to be relegated with lower priority to later times. The inputs to the proposed fiber dosimetry model would provide the capability to quantitatively address the different fiber distributions that occur at different sites, and as described in Section 4.2 above, will also afford a

systematic approach to comparative potency determinations. The experimental data proposed in the other projects will provide significant additional opportunity for model verification. We disagree that the model should not be extended to mice, as dosimetry can assist in understanding the responses observed in studies of mice, which are important in understanding specific questions relating to mechanisms and susceptibility.

- *(Dr. Mossman): There seem to be several "holes" in the development and use of an MPPD model to predict fiber deposition and risk assessment calculations, and it is unclear how related models using PM have been essential to risk assessment calculations. Given the complexity of fibers and particles in the LA sample and the need to create a model, upgrade the software, etc. (Specific Aims 1-6), this project and the proposed deadline are questionable.*

NHEERL Response: The MPPD model used for PM is proposed because its basic structure, i.e., the species-specific anatomical structures (e.g., airway architecture, surface areas) and ventilation rates have already been accepted and relied upon in the regulatory arena. For example, simulation exercises using the MPPD model to predict different dose metrics for particles (e.g., mass versus number of particles in a region, normalized by various factors such as surface area, wet tissue, etc.) were used to draw inferences and provide mechanistic insights for deliberations used to support promulgation of the National Ambient Air Quality Standard (NAAQS) for PM. The MPPD model has also been used in exercises for risk assessment of exposures of inhaled air toxics that occur in the ambient air as aerosols, as well as for safety assessment exercises of inhaled pharmaceuticals.

The “holes” alluded to in this comment are in fact what will be filled by this project. The project will first “fill” them by coding in previously peer reviewed and published algorithms that address fiber inhalability and deposition efficiency as a function of aerodynamic properties for various fiber bivariate (i.e., length and diameter) distributions. The experimental data will then be used to derive LA-specific parameters such as clearance rate constants. Because the algorithms are already published and the software upgrade will be addressed by a dedicated programmer, the proposed timeline is completely reasonable.

- *(ATSDR (Dr. Wheeler)): Should the results of modeling internal dose result in an under- or over-estimation of toxicity in the same direction that the poor exposure data in the epidemiology has caused error, the results could magnify the error. At present there appears to be too many variables for which little data is available to make internal dose calculations without wide ranges of uncertainty.*

NHEERL Response: We strongly disagree. If anything, the anatomical structures, ventilation rates, and experimentally-derived data (e.g., deposition efficiency in a specific lobe) that will be readily incorporated into the mechanisms described in the proposed human and rodent dosimetry models can only help to improve the accuracy of resultant risk estimates for dose-response analysis in each species. As described above, empirical models as currently used to address the epidemiological data have no way to systematically interrogate their structures to ascertain error components. In contrast, the MPPD model, by virtue of its mechanistic approach, is readily amenable to sensitivity analysis. For example, Monte Carlo techniques can be used to evaluate variability of individual input parameters and the MPPD structure itself is stochastic.

5. *In Vitro* Dissolution Assays

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. 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.

5.1. Necessity of Dissolution Assays for Risk Assessment.

Two reviewers raised the general issue of whether the *in vitro* dissolution assays would prove to be necessary or useful for cleanup decisions.

- *(ATSDR (Dr. Dyken)): These studies will be useful for comparative purposes and would supply information needed for model development. However, it is already known that amphiboles in general dissolve much more slowly and thus are much more biopersistent than, for example, chrysotile asbestos. It is questionable whether new findings on dissolution rates specific to Libby amphibole would significantly alter cleanup decisions.*

- *(LATAG): Comment [GH81]: Not necessary, since LA should be durable, and test methods are not agreed by all as stanstard.*

NHEERL Response: The dissolution parameters of LA have not been defined. We believe that real measurements are necessary not only for the dosimetry model but also for the risk assessment of LA and the naturally occurring asbestos samples.

5.2. Validation and Utility of I*n Vitro* Dissolution Methodologies.

Several reviewers raised the issue of whether the *in vitro* dissolution methodologies had been sufficiently validated for the types of fibers to be studied, or for samples of mixed mineralogy and morphology. Dr. Warheit also questioned whether *in vivo* biopersistence would be preferable to these *in vitro* measures of dissolution.

- *(Dr. Warheit): The proposed in vitro dissolution studies are less problematic when compared to the proposed in vitro toxicity studies, but still present a challenge in terms of accuracy. While it is widely regarded that the results of in vitro dissolution studies with biosoluble man-made vitreous fibers generally correlated with the findings of inhalation studies in rats on the same-fiber types, the in vitro dissolution methodologies have not been sufficiently validated for other fiber-types, such as amphibole asbestos. Although the in vitro methods may prove to be useful, there is no doubt that an in vivo biopersistence study in the lungs of rats would be preferable.*

- *(Dr. Masten): Dissolution studies should be conducted with LA first, as there will [be] opportunities for methods refinement during the course of these studies. Though there is much data to compare to in the literature and the NHEERL database, it is reasonable to expect more difficulty conducting and interpreting these studies with a sample with mixed mineralogy/morphology. Do the in vitro dissolution studies on LA and other fibers previously conducted by USGS supplant the need for new studies in any way?*

- *(LATAG): Comment [GH89]: [Regarding the AAL test]: Why? What is risk, need, priority, use in quantitative risk assessment? Comment [GH90]: [Regarding the SLF test]: Do you have a standard set of conditions that closely mimic that of human lungs?*

NHEERL Response: We agree that *in vivo* biopersistence studies are essential; these have been proposed to be conducted in both the inhalation and instillation studies. We will calibrate the results of the *in vitro* dissolution studies with the *in vivo* studies. Synthetic lung fluid leaches of Libby amphibole will probably not yield significant amounts of dissolution. We will most likely only do the synthetic lung fluid leach at the longest time point, 90 days, using fluids which simulate both extracellular and intracellular environments. The bulk of the dissolution tests will likely be done with the acid-accelerated leaching fluid, which is more appropriate for durable fibers. In regards to the previous USGS dissolution studies, those were not calibrated to either intratracheal or *in vivo* inhalation as proposed in this program. There is an opportunity for a collaboration to combine both agencies' data in another paper specifically for Libby amphibole, including the *in vivo* data.

5.3. Utility of the NHEERL Fiber Database.

Dr. Mossman questioned whether the intratracheal and intrapleural approaches predicted accurately different responses to different fiber types, and also whether the reexamination of the Stanton hypothesis will add significant information to what has already reported.

- *(Dr. Mossman): Although comparisons to the existing NHEERL animal database by Coffin's studies from 1978-1995 consisted of intratracheal and intrapleural injection studies, it is difficult from the papers published to determine if these approaches predicted different responses to chrysotile vs. amphiboles or fiber size as has been shown in inhalation studies by Davis. Because of their physiologic relevance and to avoid artifacts in clearance of fibers due to bypassing normal mucociliary clearance as seen with injection studies, it would be nice to prioritize inhalation studies over intratracheal/intrapleural injection studies to examine in vivo fiber dose potency in both malignant and nonmalignant lung/pleural diseases in animals. There have been several papers that have reexamined the Stanton hypothesis published already using well-characterized samples of asbestos, and I am not sure how the proposed work will add to these arguments.*

NHEERL Response: We agree that inhalation study comparisons of LA with the amosite positive control should have priority in examining fiber dose potency. However, the inhalation studies will be limited in scope. In the comparison of UICC amosite to ferroactinolite (two amphiboles with quite different fiber size distributions), both intratracheal and intrapleural exposures (Coffin *et al.* 1992) result in somewhat similar numbers of pleural and lung tumors on the basis of mass dose but not on the basis of fiber numbers in the exposures. When dose is based on post-exposure changes in tissues observed for the ferroactinolite (Cook *et al.* 1982), the two samples appear to be equipotent based on fiber numbers, especially if thin fibers of all lengths are included. These and other data suggest that amphibole fibers may be basically equipotent if *in vivo* dose changes and fiber size/morphology differences are factored into the fiber dose-response relationships. Structure based relative potency descriptors can also account for the observed differences in conventional dose-response measurements. This is the primary purpose for utilizing the database. The database can also be used to interpret results from

reported inhalation exposures and this application should become more precise as a result of development of the dosimetry model featured in this proposal.

Stanton *et al.* did not propose a hypothesis that long, thin fibers could be relied upon to describe differences in fiber carcinogenicities; only that this size category provided the best correlation between fiber number and pleural sarcoma probability in rats. Wylie et al. (1987) reported that a TEM reanalysis of some of the amphibole samples included in the Stanton study indicated differences in fiber numbers and that factors in addition to fiber length and width were likely important. Our EPA data include a much larger set of the Stanton samples including almost all of the amphibole samples, and will result in improved fiber number and size data that will likely have some impact on correlations. Finally, we will attempt to model the improved Stanton data using alternative non-linear dose-response models.

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

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.

6.1. Correlation of *In Vitro* and *In Vivo* Toxicology Results and Utility of *In Vitro* Tests.

In addition to Dr. Warheit (who also had concerns about the timing of *in vitro* tests – see comment in Section 2.2), other reviewers were concerned about the utility of *in vitro* toxicity studies and their correlations with *in vivo* studies.

- *(Dr. Castranova): Numerous parameters will be evaluated in epithelial cells and macrophages exposed to fiber samples in vitro. The list of endpoints is extensive and is an impressive display of EPA's expertise. However, such an extensive effort for in vitro studies may be misplaced. The IARC Workgroup on fiber toxicity found that in vitro data were of limited use in evaluating human carcinogenic risk to fibers (IARC Monograph on the Evaluation of Carcinogenic Risk to Humans Vol 81 Man Made Vitreous Fibers, 2002). In addition, the ILSI testing strategy for fibers sponsored by EPA (Bernstein et al. Testing of fibrous particles: short-term assays and strategies. Inhal Toxicol. 17: 497, 2005) noted that "current in vitro test systems have limited usefulness or hazard identification for characterization of dose-response" because in vitro toxicity testing "cannot evaluate biopersistence, . . . uses high doses, . . . and uses a mass rather than fiber count dose metric".*

- *(Dr. Castranova): To be time and cost efficient, limit in vitro toxicity analysis. Perhaps one should concentrate on the synergy of effects of fibrous and non-fibrous particles in the Libby Sample. A design could include evaluation of toxicity of fibrous tremolite, non-fibrous particles,*

and the combination of fibrous and non-fibrous particles. Such a design could provide mechanistic insight into risk assessment of those exposed to the mixed Libby particles.

- *(LATAG): In vitro toxicity; cellular responses are again interesting but are also difficult to extrapolate and predict human responses, so the study should also be relegated with lower priorities to later times.*

- *(Dr. Masten): The in vitro studies with human primary cells will generate interesting data but their value as part of the overall plan is unclear. Will they be used to guide the design of the in vivo studies or used in dosimetry model development? If they are meant only to inform mechanism, how does each assay/endpoint relate to a particular adverse effect (e.g. cancer, asbestosis, autoimmune). Without parallel rodent cell culture studies, can they be related to the in vivo rodent data (e.g. inflammatory, oxidative stress markers)?*

NHEERL Response: Although Drs. Warheit and Castranova cited several publications in which *in vitro* approaches were not especially useful, they could have just as easily cited many other publications in which this approach provided important information for screening compounds quickly and inexpensively, and in which *in vitro* results were predictive of both animal and human *in vivo* responses. NHEERL scientists have spent the better part of a decade developing *in vitro* methodologies that meet these latter goals and have successfully applied them to studies which compared the relative toxicity of different size air pollution particles collected from numerous geographical locations. It is our intention to apply these same methodologies to LA.

6.2. Cells to Test *In Vitro*.

LATAG questioned the types of cells to study and recommended experts to consult for advice on *in vitro* experiments. Dr. Masten asked whether parallel animal cell cultures will be conducted in order to relate assay endpoints to the *in vivo* animal studies.

- *(LATAG): Comment [GH97]: [Regarding cell culture systems]: Experts in these assays, such as Dr. Mossman or Kane, can judge which are best to interpret results for risk assessments.*

- *(LATAG): Comment [GH99]: [Regarding epithelial cells]: Not the main target tissue of LA, so little use in risk analyses.*

NHEERL Response: Experts such as Dr. Mossman have used epithelial cells extensively in studies of asbestos fibers *in vitro*. Dr. Mossman was a peer reviewer of this document and expressed an overall enthusiasm and support for the series of projects. Epithelial cells are the first cells in contact with fibers which deposit in the respiratory tract. They also outnumber alveolar macrophages by orders of magnitude. Since fibers do not have to be internalized by a cell to be toxic, it is essential to study epithelial cells as they determine the initial response to the fibers (e.g. uptake, clearance, initiation of inflammation). However, we also propose to study the effects of particles on alveolar macrophages, since these cells also play an important role in the lung's response to fibers such as LA.

- *(Dr. Masten): The in vitro studies with human primary cells will generate interesting data but their value as part of the overall plan is unclear. Will they be used to guide the design of the in*

vivo studies or used in dosimetry model development? If they are meant only to inform mechanism, how does each assay/endpoint relate to a particular adverse effect (e.g. cancer, asbestosis, autoimmune). Without parallel rodent cell culture studies, can they be related to the in vivo rodent data (e.g. inflammatory, oxidative stress markers)?

NHEERL Response: In the past we have not seen very many differences in responses of cells of different species; indeed differences in cell culture methodologies result in much larger differences. We are currently considering whether cultures of other animal cell types will be necessary

6.3. Specific Assays to Use and Samples to Test with *In Vitro* Toxicology.

Reviewers had several comments and recommendations about the types of samples to test and the assays to use in the *in vitro* toxicology approaches.

- *(Dr. Masten): Fiber size- and shape-dependent disruption of macrophage function seems particularly worthwhile, as well as effects on signaling/gene expression in mesothelial cells. Microarray analysis is semi-quantitative but not necessarily useful for deriving potency.*

- *(LATAG): Comment [GH109]: [Regarding microarray as a "powerful approach"]: No – not powerful since not useful as written for risk assessment. If EPA had human lung cell genotypes and phenotypes that correlated to susceptibility of disease, then these data and new results could be useful, but there is no indication of such capability written here.*

- *(Dr. Castranova): To be time and cost efficient, limit in vitro toxicity analysis. Perhaps one should concentrate on the synergy of effects of fibrous and non-fibrous particles in the Libby Sample. A design could include evaluation of toxicity of fibrous tremolite, non-fibrous particles, and the combination of fibrous and non-fibrous particles. Such a design could provide mechanistic insight into risk assessment of those exposed to the mixed Libby particles.*

Response: We agree that macrophage function and gene expression in mesothelial cells are very useful assays with direct consequences for fiber toxicity. As resources permit, we will study mesothelial cells and fibroblasts which are key in the development of mesothelioma and fibrosis. Although micro-array analysis is semi-quantitative when applied to the expression of individual genes, it is a very powerful and accurate approach when used to define cellular processes or pathways that have been altered by toxicants. We find this approach to be far more useful and applicable to risk assessment than just focusing on the expression of a few isolated genes. While we agree with Dr. Castranova that the *in vitro* methodology he indicates would be able to provide mechanistic insight, others (e.g. Drs. Mossman and Holian) are currently addressing this issue. We believe that the primary utility of *in vitro* testing in this project is to provide a rapid screen of many different samples (both complete and fractionated) that could be used to inform the animal studies.

7. Comparative Toxicity in Rats

Intratracheal instillation studies will provide data on LA for a ready means of comparing different types of testing materials to aid understanding of its relative biopersistence and

potential potency. 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 mucociliary transport and translocation. Initial rate estimates for these clearance mechanisms will serve to initially refine the model algorithms and additionally be used to verify 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 in special experiments to inform considerations of comparative species sensitivity.

7.1. Ambitious Nature of the Project.

As mentioned in Section 2.2, Dr. Castranova stated that the *in vivo* instillation studies "are far too ambitious", and suggested limiting studies to rats since mice are less sensitive to fibrosis. Dr. Henningsen also stated that testing of rats is sufficient.

- *(LATAG): Comment [GH110]: Omit mice, rats are sufficient as animal models, or could add a higher order mammal if wish.*

Response: We have taken this concern very seriously, and as a result we have revisited the specific aims and defined the priority order of completion. The specific aims one through five are now listed in priority order based on resources available. The first two aims (addressing the dose response relationships for single and multiple exposure scenarios and the comparative studies with other naturally occurring asbestos samples and positive and negative controls) remain the same, although we will only use rats (see below). Specific aim 1 will emphasize detailed characterization of early biomarkers and late fibrosis potential. The translocation study (specific aim 3) will focus only on LA fibers and one instillation protocol, and is now the third priority, the neonatal and timed pregnant exposure studies are now in the fourth aim, and the susceptibility study is now the fifth aim (and lowest priority).

Originally, we planned to include mice and rats both for dose-response and comparative toxicity evaluations based on the fact that the mouse toxicity database will be useful for further molecular analysis as the antibodies and genetically modified strains are easily available with the mouse models. Mice are useful for addressing issues of immunotoxicity, mechanisms, extrapolation, and potentially amelioration and treatment. Because the project may be overly ambitious and given the time constraints, we will eliminate the use of mice from all but the third and fifth specific aims. Given the availability of resources, specific aim 3 will include a single instillation and temporal particle deposition analysis in rats and possibly mice. Furthermore, relative susceptibility of Apo E-/- (knockout) mice to healthy Apo E+/+ will be conducted in specific aim 5 at a lower priority based on availability of resources.

7.2. Utility of Single and Repeated Instillation Studies (Specific Aims 1 and 2).

Reviewers had several overall comments about the strengths and weakness of the approaches outlined in Specific Aims 1 and 2, and had several suggestions about the conduct of these studies.

- *(Dr. Warheit): As discussed above, the strength of the action plans lies in the generation of the in vivo data, assessing the effects of Libby amosite asbestos fibers in the lungs of exposed rats. Although the intratracheal route of exposure studies are not as physiologically relevant as the inhalation model, the i.t. model provides an excellent first step, as a prerequisite for the generation of inhalation studies. … With respect to timing, as discussed above, it is recommended that the proposed in vitro toxicity studies should be conducted after completion of the in vivo toxicity tests. Moreover, the impact of the in vitro dissolution tests would be enhanced if compared and validated with results of in vivo dissolution tests (this could include a multidose intratracheal instillation study with several postexposure sacrifices time periods, followed by lung digestion studies).*

- *(LATAG): [Regarding instillation studies]: Comment [GH111]: Artificial exposure to bolus of unevenly distributed fibers. Comment [GH112]: No! not realistic deposition as with inhalation, so cannot make such extrapolations and data are too uncertain to use in risk assessments. Anomalous data and unrealistic results could be obtained from bolus doses vs more uniform lower steady state exposures by inhalation … Comment [GH118]: Artificial unrealistic exposures.*

- *(Dr. Masten): The proposed intratracheal studies are not providing much information that could not be obtained from inhalation studies of equivalent or shorter time course. Given the cost and time to set up the inhalation exposure facility for the LA subchronic study, there would be only incremental cost to do additional inhalation studies instead of intratracheal installation.*

- *(LATAG): Comment [GH120]: Why this length? Why not a more uniform standard comparable time of 90 days?*

- *(Dr. Castranova): The design calls for evaluation of pulmonary responses 1 day to 4 months post-exposure, except for one group of rats where mesothelioma would be evaluated 1 year post. I suggest monitoring all pulmonary responses up to 1 year post as well.*

Response: We agree that the proposed instillation studies are an important component of the overall comparative toxicity assessment of LA. We agree that *in vivo* dissolution (biopersistence of fibers) should be compared to *in vitro* dissolution, and we plan to conduct these studies, as emphasized in the revised NHEERL plan. Regarding the use of inhalation rather than instillation, we have only limited resources to perform comparative toxicology. Inhalation studies provide realistic exposures, but are extremely time-consuming, expensive, and require large amounts of sample. Therefore, only a limited number of inhalation studies can be conducted for specific risk assessment purposes. In contrast, instillation studies require only small amounts of sample, provide lower dose variability, precise dose delivery, and allow effective comparisons at multiple doses and times. The revised repeated instillation protocols now include time points of 3 months (13 weeks) to match the length of the subchronic study. We will also examine all responses in rats 1 year after exposure. We will also explore the feasibility of holding groups of rats for up to 2 years after exposure in order to assess possible tumor development. We agree that intrapleural administration may be useful, and we will consider these experiments if time and resources allow.

7.3. Utility of Translocation Studies and Tissue Fiber Burden Determinations (Revised Specific Aim 3).

Two researchers questioned the utility of the translocation study in light of other components of the research program.

- *(ATSDR (Dr. Dyken)): Translocation studies. These studies may be useful to answer basic research questions, although researchers have already shown translocation of amphiboles. It is questionable whether new findings on translocation specific to Libby amphibole would significantly alter cleanup decisions.*

- *(Dr. Masten): Fiber translocation can be evaluated as part of the subchronic inhalation study. Why is a separate study proposed?*

NHEERL Response: We agree that translocation has been demonstrated before, but the specific parameters associated with LA have not been measured. These results will be important for the development of the dosimetry model. We recognize that these data are not as high a priority as other instillation studies and this is reflected in our prioritized list, where translocation studies are third in importance of the five studies proposed.

Dr. Castranova questioned the choice of tissue samples from the intratracheal studies to study for fiber burden analysis.

- *(Dr. Castranova): [Under In Vitro Dissolution section]: Fiber count and fiber dimension analyses in lung, bronchial-associated lymph nodes, and pleural tissue is planned for the inhalation study. It should also be a component of the intratracheal installation experimental design. Pleural content is currently lacking in the IT study design.*

- *(Dr. Castranova): [Under Intratracheal Instillation Studies]: As mentioned, previously measurement of fiber count and fiber dimension in lung, bronchial – associated lymph nodes, and pleural tissue is essential.*

NHEERL Response: Given the nature of delivery represented by the intratracheal study and due to cost constraints, we proposed to limit the fiber burden analysis to exposure time points (0, 1.5, 3, 6 and 24 hours and 1 week) in the trachea, lung lobes and GI only. This will allow initial estimation of mucociliary clearance rates for the dosimetry model that can be verified with the inhalation data. Because the inhalation project will provide pathology data for correlation with the estimates, we instead devoted a more comprehensive determination of tissue burdens in that study.

7.4. Utility of neonatal and timed pregnant rat exposures (revised Specific Aim 4).

Dr. Henningsen questioned the utility of neonatal rat exposures, and stated that we should just assume they are more sensitive and apply an exposure or uncertainty factor.

- *Comment [GH114]: Why? What is rationale? Relative susceptibility of young vs adults may be of interest if the latency period or severity or target tissues change; else, just assume young are more sensitive and adjust with exposure factors and safety or uncertainty factors to protect.*

Application of an exposure or uncertainty factor is probably not a safe assumption. Inappropriate application of an additional safety factor could drive the cleanup efforts, as noted

in comment 7.4 (Dr. Dyken), to the "limit of economic feasibility". The specific aim 4 originally included maternal exposure as well as LA translocation studies. As mentioned before, in the revised proposal the translocation study is now in specific aim 3. The proposed study of exposing pregnant rats and determining the likely pathological and fetal lung developmental effects will be done only if the resources are available. Similarly the exposure of neonatal Wistar Han rats and subsequent inflammation and fibrosis development will be studied at several time points up to 1 year. Developmental exposures often lead to different outcomes than exposures of adult animals. Based on the general comments received from the reviewers, the developmental and neonatal studies are placed at a lower priority even though we believe that these studies will provide important information on special susceptible subgroups.

7.5. Utility of Susceptible Disease Models (Revised Specific Aim 5).

Several reviewers had remarks on the choice and utility of specific animal susceptible disease models.

- *(ATSDR (Dr. Dyken)): Studies on more-sensitive disease outcomes. These studies may be useful for basic health intervention research. However, it is questionable whether findings would practically influence cleanup levels at Libby. Cleanup levels selected based on pulmonary endpoint-based risk may likely be near the limit of economic feasibility.*

- *(LATAG): [In response to the title of the Specific Aim addressing animal susceptibility models]: Comment [GH126]: So what? You are using fairly homogeneous strains of inbred rats; whereas the Libby humans are heterogeneous and no obvious patterns or causes of susceptibility are known. However, the most useful clinical data on such results lies dormant in Libby at the CARD clinic, awaiting funding of this highest priority study for use in risk analyses.*

- *(Dr. Castranova): The issue in Libby, MT and El Dorado, CA is asbestosis, lung cancer, and mesothelioma. This being the case, why evaluate cardiovascular – compromised models or exposure to the fetus? The project will be large enough if limited to pulmonary responses.*

- *(Dr. Masten): For evaluating the potential to cause autoimmune disease and mesothelioma, use of an appropriate animal model (e.g. Brown Norway rat) is the preferred approach. For evaluating genetic susceptibility, there should be clear (mechanistic) questions to address, not just selection of familiar models. What is the relationship of hypertension and atherosclerosis to asbestos induced health effects? How will these models shed light on susceptibility to mesothelioma?*

Response: Human chronic cardiovascular and pulmonary diseases share common risk factors such as increased systemic inflammation, oxidative stress, increased microvascular thrombosis, and generalized immunosuppression. The spontaneously hypertensive (SH) rats which are prone to hypertension and cardiovascular disease exhibit similar risk factors common in chronic pulmonary disease. We will also examine a mouse model of atherosclerotic disease (ApoE knockout mice and their control background strain). In a number of studies NHEERL has demonstrated that SH rats are more susceptible to particulate matter and tobacco smoke-induced pulmonary injury and inflammation. NHEERL recently reported that the airway inflammatory response to high level sulfur dioxide exposure in SH rats is ten times more pronounced than the response seen in normal Sprague Dawley (SD) rats. It is apparent that the toxicants which

deposit in the airways will cause greater toxicity in SH rats than in WKY rats. Nearly one third of the human population suffers from increased systemic hypertension, and it is likely that those individuals have remarkable susceptibility to pulmonary inflammation in response to LA exposure relative to healthy individuals. In an NHEERL study (in preparation), we showed that the parental Wistar Kyoto (WKY) rats develop granulomatous changes following particulate matter exposure, analogous to those seen in Wistar rats exposed to asbestos fibers (Bernstein et al., 2006). It is possible that granulomatous/fibrotic changes seen in residents of Libby are similar to those seen in WKY and Wistar rats and that SH rats with added sensitivity to inflammation may have an even greater response. The opportunity to conduct this study will provide insights into further epidemiological evaluation of Libby residents to determine relationships between occurrence of cardiovascular disease and asbestos-induced pulmonary inflammation and granulomatous changes. Thus, we propose to study relative susceptibility of WKY and SH rats and ApoE knockout mice to LA fibers using an exposure regimen similar to that proposed in the first specific aim. This study again will be conducted based on the priority as set in the proposal.

Some studies have used autoimmune-prone New Zealand mixed (NZM) mice which produce autoantibodies (Brown et al., 2005), and normal C57Bl6 mice also produce these markers of autoimmunity (J. Pfau, personal communication). What we have proposed is to archive sera from the proposed mouse studies and determine if any of those samples test positive for autoimmune antibodies. If these antibodies are detected, it will be important to follow up these findings with new proposals to further investigate autoimmunity. These studies could be done in consultation with Dr. Jean Pfau at the University of Montana who has conducted these types of studies.

8. Inhalation Toxicology in Rats

This project provides data on the relative potency of inhaled Libby amphibole (LA) compared to UICC amosite, a known fibrogenic 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., translocation rates) and accurate retained dose predictions.

In general, the proposed project design was viewed favorably:

- *(ATSDR (Dr. Dyken)): Animal inhalation studies. These studies could provide data that would directly assist in setting cleanup levels, since exposure would be based on air levels.*

- *(Dr. Castranova): The experimental design is logical and the project scope achievable.*

8.1. Choice of Rat Strain in Studies.

Two reviewers had suggestions for the choice of rat strain to be used in the inhalation project.

- *(LATAG): Subchronic inhalation in rats – use Wistar rats, and not the F344 strain, since Wistars have less confounding cancer and other diseases. Comment [GH130]: No! do NOT use this strain, since their spontaneous lung lesions are excessive for LA studies. Comment [GH135]: Use Wistar rats, not F344.*

- *(Dr. Masten): We recommend using Wistar Han rats. The NTP has recently switched to this animal model for all NTP studies (http://ntp.niehs.nih.gov/go/29502). This is important if the subchronic data is to be used in designing NTP chronic studies. The Wistar Han has been used in previous fiber studies (e.g. Bernstein et al. Inhal. Toxicol. 2005).*

NHEERL Response: Since a large fibrosis and mesothelioma database is available for a variety of asbestos fibers in Fisher (F344) rats, we had originally planned to use this strain to study LA. Since the primary question to be addressed in the proposed studies is the fibrotic potency of LA, there were extensive discussions on the appropriate rat strain to be used for this purpose. NHEERL previously demonstrated that the fibrosis induced by combustion particle exposure is much greater in Sprague Dawley (SD) rats, which have greater fibrosis than F344 rats, which in turn have greater fibrosis than Wistar rats, despite the inflammatory response being greater in Wistar rats. We have also shown that Wistar based Wistar Kyoto (WKY) rats do not develop as pronounced fibrosis as SD rats following particulate matter exposure but exhibit marked inflammatory response and airways disease. Thus, it is possible that although not as remarkable as fibrosis in SD rats, Wistar rats may readily develop granuloma/fibrotic lesions due to their greater inflammatory response (as demonstrated by Bernstein, Inhal. Toxicol. 2006). Another important consideration is that the dosimetry model is based on the Long Evans and also the F344 rat in the near future. The large database on F344 is a compelling reason to perform comparative evaluations of LA using this rat strain. If the NTP wants to use Wistar rats then they can do a 90-day sacrifice and we can compare responses at that time point. The Baseline Risk Assessment is likely to go forward on the basis of the NHEERL work sooner than on the basis of the NTP chronic exposure study. We would appreciate further discussion and dialogue on the spontaneous lung lesion rates in F344 rats, but consideration of all of other factors leads us to the conclusion that we will maintain our choice of the F344 rat for the inhalation studies.

8.2. Range finding study (Specific Aim 1).

Two reviewers had concerns regarding the duration and design of the proposed range finding study for this project. Dr. Masten stated that the proposed 5 day range-finding study may not be optimal; later discussions in a conference indicated that he believed a 2 week study would be appropriate. Dr. Henningsen questioned how the proper concentration of fibers will be determined, and which fibers will be measured

- *(NIEHS / NTP): The proposed 5 day range-finding study may not be optimal for designing subchronic studies. Lung burden is critical for setting doses in subchronic studies and need some clearance time to get an idea of biopersistence. We recommend more dose groups (5). It might be useful to include amosite in the 5 day study as well.*

- *(Dr. Castranova): The design calls for range finding studies. Data from IT exposure could be useful in defining the range of aerosol concentrations to be tested. For this reason, the*

intratracheal instillation studies should be scheduled six months to a year before the inhalation studies, so that useful data are available.

- *(LATAG): [Regarding determining proper concentration]: Comment [GH132]: How? Fiber#, structures, mass, surface area? Define and standardize for doses. [Regarding the fibers that will be measured]: Comment [GH134]: What about the smaller size fraction that is associated with fibrosis? They should also be measured and assessed for toxicity, as well as fragments.*

NHEERL Response: To account for clearance in the range finding study, we shall add groups of animals sacrificed after 2 weeks of exposure in addition to 5 days of exposure. The experimental design will be revisited to see if we can accommodate an amosite control group within the expected budget. We agree with Dr. Castranova that instillation studies could be useful in defining the range of concentrations to be used with the inhalation study. It is likely that some of the instillation studies will be completed in time to allow this use. Regarding LATAG's comments, we will measure all of those properties of fibers, and define them for the different concentrations to be used in the exposures. We will also measure all size structures and fibers collected in the exposure atmosphere.

8.3. Subchronic 90-day study (Specific Aim 2).

One reviewer questioned the duration of the 90-day study as insufficient.

- *(LATAG): [W]hat is the purpose for only exposing rats to Libby amphibole for 90 "partial" days (only 15 days of continuous exposure divided over 90days!), when the spectrum of asbestos related diseases (ARD) are thought to occur after chronic "continuous" (likely intermittent excursions) exposure with varying latency times; thus, subchronic exposure may miss critical disease endpoints of relevance to Libby amphibole. … Comment [GH129]: Again, this only amounts to about 15 total days of cumulative exposure, so it is NOT a truly continuous subchronic study. [Regarding 1-year post-exposure sacrifice time] … Comment [GH131]: Good but limited by the short time and low amount of exposure to LA, and will have little use for risk assessment – except for possible subchronic fibrotic endpoints, if exist.*

NHEERL Response: A six-hour per day exposure is standard operating procedure for nose-only exposures (no institutional animal care and use committee would allow a longer nose-only exposure period). LATAG appears to be adding the exposure times together to arrive at a cumulative exposure time. Using this method, a standard 2 year chronic study would amount to only 4 months of exposure. The exposure concentrations used in a nose-only exposure are designed to be high enough to account for the intermittent nature of the exposures. Ninety day studies are the standard in mammalian toxicology testing for covering a sufficient period of the animal's life to predict target organs and approximate potency for repeated exposure over a considerable portion of their lifetime. An important consideration is that we will hold groups of animals for at least 1 year after the cessation of the exposure (and, if resources permit, up to 2 years after exposure) to allow for development of pathological responses following exposure.

A question was raised about the use of cell proliferation assays.

- *LATAG: Comment [GH136]: How will this be used for risk analyses? Please justify, or else it will have little use.*

NHEERL Response: The BRDU assay is an index of cell proliferation. If fibroblasts are proliferating, this will be a useful marker for the onset of fibrosis.

A question was raised about the assessment of fiber burden in the organs of rats exposed to LA during the subchronic exposure.

- *Comment [GH137]: OK, but since the fiber loading is DYNAMIC, then the timing is critical, and these tests should be done on serially sacrificed animal models to evaluate bio-persistence and translocation.*

NHEERL Response: As indicated in this section, we will assess fiber burden in rats exposed for 1 month and 3 months, and rats exposed for 3 months and sacrificed 3, 6, and 12 months post-exposure.

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DEPARTMENT OF HEALTH & HUMAN SERVICES
Public Health Service
Centers for Disease Control and Prevention
National Institute for Occupational Safety and Health

Memorandum

Date: May 9, 2007

From: Vincent Castranova

Subject: Review of EPA NHEERL Research Plans for Libby Asbestos

To: Dr. Stephen Gavett

Description:

The research program consists of five projects:

1. Dosimetry model development and simulation studies – create, implement and upgrade models for deposition and clearance in rats, humans, and mice.
2. *In vitro* dissolution assays – monitor dissolution, leaching and splitting/breakage in synthetic lung lining fluid and acid solution.
3. Use of *in vitro* toxicology to compare the potency of different test materials – using primary human tracheobronchial cells and alveolar macrophages, measure particle effects on signaling pathways, mRNA profiles, gene expression, cytokine production, phagocytosis and ROS production by macrophages, mucin and cilia of epithelial cells, viability, oxidant stress, DNA damage, and genotoxicity.
4. Comparative toxicology in mice and rats – determine the dose-response and time-course of pulmonary reactions to intratracheally instilled fiber samples. Use adult, neonatal, and cardiovascular – compromised rodent models. Also expose pregnant rats and determine effects on the fetus.
5. Inhalation toxicology in rats – conduct a subchronic 90 day exposure and monitor fiber burden, clearance, and pulmonary responses up to 12 month post-exposure.

A. Fiber samples:

Two samples of Libby amphibole will be studied (a sample collected in 2000 for *in vitro* and IT studies and a sample prepared in 2007 for inhalation studies). Positive control fiber samples would be UICC standard chrysotile, UICC amosite, UICC crocidolite, or refractory ceramic fibers. Negative control fiber samples would be wollastonite or glass wool. Fiber samples will be characterized for fibers/mg and delivered using a mass metric.

Problems:

1. There is a rush to conduct toxicology (*in vitro* dissolution, *in vitro* toxicity, and IT and inhalation studies are scheduled to start by Y 07 Q4). Due to the urgency to begin

studies, little attention is given to sample preparation, which is critical to program success.

2. The Libby and El Dorado amphibole exposures are most like tremolite in chemical nature. Therefore, chrysotile and refractory ceramic fibers would be poor positive controls. Amosite would be satisfactory, but fibrous tremolite would be the most relevant positive control.
3. There is danger in using 2 Libby samples collected and prepared at different times (in 2000 vs 2007). It is unlikely that the two samples would be identical in fiber count/mg; types of contaminating particles, fiber dimension, etc. It would be preferable to prepare and well characterize a single Libby sample to be used for all projects throughout the program duration.
4. I have seen electron micrographs of Libby amphibole samples, i.e., the Libby six mix. Fibers tend to be relatively short (<10 μm), with few fibers between 10 – 20 μm. In addition, samples are contaminated by a significant amount of non-fibrous particles. In the study design, the potency of the Libby sample would be compared to UICC standard fiber samples, which are rich in fibrous particles of relatively long size (10 – 20 μm). Therefore, the Libby sample is likely to be less toxic. Studies conducted by the toxicology group at the University of Montana support this premise.
5. I suggest that a major effort be placed in preparation and characterization of fiber samples.
    a) Prepare a single Libby sample to be used in all studies. Characterize the fiber count/mass, the fiber length distribution, the amount of non-fibrous particles/mass, and the composition of non-fibrous particles.
    b) The design states that effort will be made to remove non-fibrous particles with an aerodynamic diameter of >10 μm. Effort should be made to remove as much non-fibrous material of all sizes as possible to enrich the Libby amphibole in the sample. This may prove very difficult. An alternative is to use tremolite fibers as the positive control. Mill the fibers to shorten the length to be comparable to the Libby fibers. Lastly, dope the tremolite sample with non-fibrous particles of similar composition and amount as the Libby sample. In these ways, the Libby sample and the positive control would be more comparable.
    c) The negative control fiber sample should be milled and doped in a similar manner.
    d) An additional particle control could be a sample of non-fibrous particles of similar size and composition to those found in the Libby sample.
    e) Wollastonite would be a more appropriate negative control fiber, since glass fibers are likely to be thicker than amphibole fibers.
    f) Berman and Crump conducted a meta analysis of epidemiological data with asbestos and lung cancer for EPA (Technical support document for a protocol to assess asbestos – related risk, 2001). In this analysis, they found that fibers > 10 μm in length were substantially more carcinogenic than those from 5 – 10 μm. This points to the importance of having similar fiber lengths in the Libby and the positive control samples.

B. *In vitro* durability and *in vivo* biopersistence:

Measurement of *in vitro* rates of dissolution, leaching and splitting/breakage are essential. However, as noted in an ILSI testing strategy for fibers sponsored by EPA (Bernstein et al. Testing of fibrous particles: Short-term assays and strategies. Inhal Toxicol 17: 497, 2005), measurement of *in vivo* biopersistence and translocation is also essential.

Comments:

1. Fiber count and fiber dimension analyses in lung, bronchial-associated lymph nodes, and pleural tissue is planned for the inhalation study. It should also be a component of the intratracheal installation experimental design. Pleural content is currently lacking in the IT study design.

C. *In vitro* toxicology:

Numerous parameters will be evaluated in epithelial cells and macrophages exposed to fiber samples *in vitro*. The list of endpoints is extensive and is an impressive display of EPA's expertise. However, such an extensive effort for *in vitro* studies may be misplaced. The IARC Workgroup on fiber toxicity found that *in vitro* data were of limited use in evaluating human carcinogenic risk to fibers (IARC Monograph on the Evaluation of Carcinogenic Risk to Humans Vol 81 Man Made Vitreous Fibers, 2002). In addition, the ILSI testing strategy for fibers sponsored by EPA (Bernstein et al. Testing of fibrous particles: short-term assays and strategies. Inhal Toxicol. 17: 497, 2005) noted that "current *in vitro* test systems have limited usefulness or hazard identification for characterization of dose-response" because *in vitro* toxicity testing "cannot evaluate biopersistence, . . . uses high doses, . . . and uses a mass rather than fiber count dose metric".

Comment:

1. To be time and cost efficient, limit *in vitro* toxicity analysis. Perhaps one should concentrate on the synergy of effects of fibrous and non-fibrous particles in the Libby Sample. A design could include evaluation of toxicity of fibrous tremolite, non-fibrous particles, and the combination of fibrous and non-fibrous particles. Such a design could provide mechanistic insight into risk assessment of those exposed to the mixed Libby particles.

D. Intratracheal instillation studies:

The design includes studies with adult, neonatal and cardiovascular – compromised rats, adult and cardiovascular-compromised mice, and rat fetuses upon in utero-exposure.

Comment:

1. The studies are for too ambitious. Both the ILSI Workgroup to develop a short-term testing strategy for fibers sponsored by EPA (Bernstein et al., 2005) and the EPA Panel for chronic fiber testing (Test Guidelines for Chronic Inhalation Toxicity and Carcinogenicity of Fibrous Particles, Sept. 2000) stated that the rat was the preferred

species for testing. In general, mice are less sensitive to fibrosis. I suggest limiting IT studies to rats.

2. The issue in Libby, MT and El Dorado, CA is asbestosis, lung cancer, and mesothelioma. This being the case, why evaluate cardiovascular – compromised models or exposure to the fetus? The project will be large enough if limited to pulmonary responses.
3. As mentioned, previously measurement of fiber count and fiber dimension in lung, bronchial – associated lymph nodes, and pleural tissue is essential.
4. The design calls for evaluation of pulmonary responses 1 day to 4 months post-exposure, except for one group of rats where mesothelioma would be evaluated 1 year post. I suggest monitoring all pulmonary responses up to 1 year post as well.

E. Inhalation study:

The experimental design is logical and the project scope achievable.

Comment:

1. The design calls for range finding studies. Data from IT exposure could be useful in defining the range of aerosol concentrations to be tested. For this reason, the intratracheal instillation studies should be scheduled six months to a year before the inhalation studies, so that useful data are available.

June 11, 2007

RE: Review of NHEERL description of research projects to address" Dosimetric and Toxicologic Assessment of Amphibole Fiber-Containing Material from Libby Montana"

Significance: This document describes proposed research over a 3 year period of funding to address the toxicology *in vitro* and *in vivo* of amphibole fiber-containing vermiculite ore from Libby, Montana that has resulted in an excess of both malignant and nonmalignant respiratory disease in workers and citizens of this community. In addition, the product was sent to other plants in the US for processing and is thought to exist in at least 30 million homes. Thus, the research proposed is highly significant as little is known about the toxicology of the Libby ore, its products, and risks to exposed populations of workers and citizens residing in homes using these products. Because of the mineralogical uniqueness and complexity of the Libby amphibole (LA), it does not fall under the regulatory sphere of the six types of 'asbestos' fibers as defined by the EPA and other government agencies. For these reasons, the studies proposed are also mandatory to determine how the biological effects and solubility of the Libby ore/products compare to the classical asbestos types.

Summary of proposed work: As stated, research will be performed in the following areas: 1) comparative *in vitro* dissolution of asbestos-fiber containing samples; 2) comparative *in vitro* cellular cytotoxicity; 3) comparative *in vivo* toxicity of asbestos fiber-containing samples (delivered by intratracheal instillation in rats and mice); 4) subchronic inhalation toxicology of the Libby material in rats and 5) dosimetric modeling of the results from the *in vitro*, comparative toxicity, and inhalation toxicology studies to provide a more informed risk assessment of the Libby asbestos material. As acknowledged, detailed protocols and key aspects of this document are lacking and will be developed and reviewed internally prior to the initiation of studies.

Feedback questions:

1) Do the proposed *in vitro, in vivo,* and dosimetry studies address the risk assessment of the Libby situation appropriately? Yes. The research plans are developed to characterize key features of the Libby Amphibole (LA) that will predict its internal dose/retention. Proposed research will characterize molecular and cellular mechanisms of toxicity and tissue reactions leading to disease endpoints in rodents. In addition, they will predict the inherent toxicity of LA in comparison to asbestos types and whether there is differential sensitivity to LA or asbestos types related to age or other pathologies. The use of well-characterized and sized positive (amphibole asbestos) and negative (nonpathogenic fibers or particles) controls is imperative as well as determining the most appropriate basis for equivalent comparisons between particulates (surface area, fiber number, fiber size) prior to the initiation of long-range or animal studies. Although the studies seem overambitious for a 3 year period, they address all of the key mechanisms of action that have been reported for asbestos including tests for durability (*in vitro* dissolution data, tissue/fiber burden studies in animals), epigenetic effects (cell signaling, cytokine production, proliferation) and genetic (chromosomal and other genetic abnormalities)

effects. Subchronic inhalation studies in rodents will complement these mechanistic studies and document disease potential.

2) Will the research projects produce data and products useful to EPA Region 8 which must provide technical support to the Libby Montana community? Yes. Hopefully, data will reveal whether or not the risk of exposure to LA reflects the same health risks and diseases seen with different types of asbestos and/or explain the unique pleural fibrosis that is seen in the Libby population that has not been observed thusfar in animal models of asbestos exposure. Moreover, studies should elucidate the components of LA that are pathogenic. Lastly, they will determine whether certain populations (the young, the elderly, compromised individuals, etc.) are more prone to LA-associated diseases.

3) Do you have recommendations for improvements that will increase the likelihood that the projects will achieve their stated goals? Yes. Although the primary focus of all studies is on the LA material (presumably the Six Mix from 6 different sites in Libby) characterized in 2000, comparing this complex mix of fibers, fragments and particles to asbestos fiber samples or other non-asbestos amphiboles may reveal little effects (as several laboratories have already reported) because of the small fiber:particle ratio. Moreover, testing may not reflect the toxicity of the product (Zonolite) used in homes. Sizing or fractionation of the LA and final product for enriched fiber:particle or elongated fiber:particle preparations would be preferable to examination of site-specific chrysotiles or taconite-associated amphiboles. Although comparisons to the existing NHEEL animal database by Coffin's studies from 1978-1995 consisted of intratracheal and intrapleural injection studies, it is difficult from the papers published to determine if these approaches predicted different responses to chrysotile vs. amphiboles or fiber size as has been shown in inhalation studies by Davis. Because of their physiologic relevance and to avoid artifacts in clearance of fibers due to bypassing normal mucociliary clearance as seen with injection studies, it would be nice to prioritize inhalation studies over intratracheal/intrapleural injection studies to examine *in vivo* fiber dose potency in both malignant and nonmalignant lung/pleural diseases in animals. There have been several papers that have reexamined the Stanton hypothesis published already using well-characterized samples of asbestos, and I am not sure how the proposed work will add to these arguments.

4) Do the funding and timelines for the research projects appear to be reasonable for the risk assessment and technical support of the Libby community, considering that they will inform the Record of Decision by EPA to be made in 2010? Questionable from the time plan and priorities presented. The first project is to develop a dosimetry model to predict fiber deposition and retained fibers in tissues of rodents and humans. There seem to be several "holes" in the development and use of an MPPD model to predict fiber deposition and risk assessment calculations, and it is unclear how related models using PM have been essential to risk assessment calculations. Given the complexity of fibers and particles in the LA sample andthe need to create a model, upgrade the software, etc. (Specific Aims 1-6), this project and the proposed deadline are questionable. While the compendium of proposed toxicologic studies is robust and likely to provide mechanistic information and disease in rodents, it is also unclear how analysis of all data will be amalgamated and incorporated into a "Record of Decision".

In summary, the proposed studies have many strengths and the credentials of the investigators listed are outstanding. Hopefully the EPA or other agencies are also initiating epidemiologic and clinical studies on the Libby population to elucidate biomarkers, enable therapeutic strategies and find risk factors in humans now afflicted with LA diseases, both malignant and nonmalignant.

Sincerely,

Brooke T. Mossman, PhD
Professor of Pathology

Review of Research Plan entitled "Dosimetric and Toxicologic Assessment of Amphibole Fiber-Containing Material from Libby, Montana

DB Warheit – May 25, 2007

Overall Research Approach

The National Health and Environmental Effects Research Laboratory (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.

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 90-day 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 (or intranasal) instillations of rats and mice, focused on intermittent exposures that are likely to be more representative of actual exposures experienced by children and residents.

NHEERL will conduct a subchronic inhalation exposure study in the rat, examining a variety of toxicological endpoints and the relationship between duration of exposure and the nature and persistence of effects.

Warheit critique

This Reviewer appreciates the urgency associated with the serious health problems resulting from exposures to amphibole fiber-containing vermiculite ore from Libby, Montana. The proposed studies represent a multidisclipined attempt to address the key areas of uncertainty related to Libby asbestos health risk assessments. Although this Reviewer was not present at the January, 2007 planning meeting in Research Triangle Park to discuss research priorities, I have only moderate enthusiasm for the proposed plan of action to conduct dosimetric and toxicological assessments of the Libby amphibole material. In this regard, the strengths of the plan of action are associated with the planned intratracheal comparative studies, followed by the 90-day inhalation studies. The weaknesses of the project are associated with the planned *in vitro* toxicity studies. In addition, it is unclear whether the *in vitro* dissolution studies will be instructive; and finally the dosimetry model, if developed properly, could be very useful but should only be applied following the generation of *in vivo* (intratracheal and/or inhalation) data. Thus, the dosimetry (deposition/clearance) data should not precede the *in vivo* data, but rather should be implemented after the generation of *in vivo* data (preferably morphometric data – which is not planned in this project) has been successfully achieved.

With respect to the proposed *in vitro* toxicity data, previous studies have reported little correlation between the relative toxicity of particles when comparing lung toxicity rankings following *in vivo* instillation compared to *in vitro* cell culture exposures (Seagrave *et al*., 2002; 2003; 2005). Moreover, a recently published study was designed to assess the capacity of *in vitro* screening studies to predict *in vivo* pulmonary toxicity of several fine or nanoscale particles in rats. The authors concluded that *in vitro* cellular systems will need to be further developed, standardized, and validated (relative to *in vivo* effects) in order to provide useful screening data on the relative toxicity of inhaled particles (Sayes *et al*., 2007). Moreover, the ILSI Risk Science Institute Working Group on testing assays for fibrous particles concluded that current *in vitro* tests systems have limited usefulness for hazard identification or characterization of dose response relationships (ILSI – Bernstein et al., 2005). In addition, it was suggested that for some fiber-types, these tests may help to identify and evaluate possible mechanisms involved in fiber-related lung pathogenesis. But this concept was viewed as supplementary to the identification of fiber-induced toxic effects by utilizing *in vivo* methodologies. Thus, it is suggested that any *in vitro* toxicity studies designed to assess hazard potential of these amphibole-like materials should only be conducted after completion of *in vivo* tests and the predictability of *in vitro* tests can only be validated using *in vivo* results.

The proposed *in vitro* dissolution studies are less problematic when compared to the proposed *in vitro* toxicity studies, but still present a challenge in terms of accuracy. While it is widely regarded that the results of *in vitro* dissolution studies with biosoluble man-made vitreous fibers generally correlated with the findings of inhalation studies in rats on the same-fiber types, the *in vitro* dissolution methodologies have not been sufficiently validated for other fiber-types, such as amphibole asbestos. Although the *in vitro* methods may prove to be useful, there is no doubt that an *in vivo* biopersistence study in the lungs of rats would be preferable.

As discussed above, the dosimetry models can provide important information, but the success of this project is dependent upon having reliable *in vivo* data. Lung morphometry data on (inhaled) amphibole fiber deposition, clearance, and pathological progression-type data would be the most useful source for developing dosimetric models. Alternatively, *in vivo* lung morphology assessments would be adequate. However, it is unclear how progress on dosimetric assessments could be advanced in the absence of generating data associated with *in vivo* lung tissue evaluations.

As discussed above, the strength of the action plans lies in the generation of the *in vivo* data, assessing the effects of Libby amosite asbestos fibers in the lungs of exposed rats. Although the intratracheal route of exposure studies are not as physiologically relevant as the inhalation model, the i.t. model provides an excellent first step, as a prerequisite for the generation of inhalation studies.

With respect to timing, as discussed above, it is recommended that the proposed *in vitro* toxicity studies should be conducted after completion of the *in vivo* toxicity tests. Moreover, the impact of the *in vitro* dissolution tests would be enhanced if compared and validated with results of *in vivo* dissolution tests (this could include a multidose intratracheal instillation study with several postexposure sacrifices time periods, followed by lung digestion studies).

Responses to Questions

Do the proposed *in vitro, in vivo,* and dosimetry studies address the risk assessment of the Libby situation appropriately?

The proposed *in vivo* studies appropriately address the hazards of Libby amphibole asbestos fibers. The utility of the proposed dosimetry studies would be enhanced contingent upon first obtaining reliable *in vivo* lung deposition, clearance and pathology data. The *in vitro* toxicity data could be utilized to test mechanistic hypotheses generated from the results of the *in vivo* studies. The *in vitro* dissolution studies should be carried out in conjunction with *in vivo* biopersistence studies.

Will the research projects produce data and products useful to EPA Region 8 which must provide technical support to the Libby Montana community?

This Reviewer believes that the current research plan needs to be revised according to the strategies outlined above. There is no doubt that successful completion of the intratracheal and inhalation studies could provide very useful data for assessing the hazards of Libby amphibole fibers in the lungs of rats. The *in vitro* toxicity studies could provide useful, hypothesis-driven, mechanistic data, but should not be utilized for preliminary screening evaluations or hazard assessments.

Do you have recommendations for improvements that will increase the likelihood that the projects will achieve their stated goals?

Please see above recommendations.

Do the funding and timelines for the research projects appear to be reasonable for the risk assessment and technical support of the Libby community, considering that they will inform the Record of Decision by EPA to be made in 2010?

The timelines for the various research projects should be reorganized as described above. It is strongly suggested that the *in vivo* studies precede the development of *in vitro* dissolution, *in vitro* toxicity and dosimetry studies. This may not be the most convenient strategy but clearly will lead to the most productive and accurate results.

Jill Dyken's comments, 5/14/2007

Comments on NHEERL Research Project Plan

This is an ambitious and thorough research plan which, if implemented fully, would add considerably to the body of knowledge of how Libby amphibole and other asbestos-related minerals contribute to disease. I evaluated the proposed research projects in light of the following five principles for setting public health research priorities:

1. The research project itself protects public health. (*e.g.*, a study of exposure mitigation strategies).
2. The research project provides information essential for making evidence-based cleanup or exposure mitigation decisions (*e.g.*, dose–disease response analysis to set appropriate cleanup levels).
3. The research project assists governmental agencies and partners in making appropriate policies affecting public health (*e.g.*, research showing regulation is needed to prevent hazardous exposures).
4. The research project serves community needs and concerns (*e.g.*, studies of disease diagnosis and treatment).
5. The research project advances the basic science upon which public health decisions are made (*e.g.*, basic science leading to understanding of disease mechanisms).

NHEERL's proposed research projects generally meet principle 5 in advancing the basic science describing how Libby amphibole materials cause disease and how the toxicity relates to other (better studied) asbestos materials. However, because of significant time and resource commitments associated with the proposed plan, it is not clear that conclusive results will be available within a time frame that would allow important risk management decisions to be made for the Libby community.

I recommend that NHEERL prioritize toxicological research based on fiber air concentrations over research based on modeled internal fiber burden. I recognize that internal fiber burden may be the more toxicologically relevant dose metric. However, given the current state of science, it does not contribute in a practical way to cleanup-related decision making for the following reasons:

- Epidemiological studies upon which asbestos risk assessments depend describe only (highly uncertain) air fiber concentration measurements;
- The relation of an animal dose to realistic human exposure dose or fiber burden is unknown and unlikely to be available in a reasonable period of time; and
- The correlation between human fiber burden and adverse health outcome is unclear and unlikely to be determined in a reasonable period of time.

A large portion of the proposed research relies on developing a model to describe internal fiber burden. While this could be useful for long term research, it is unlikely to contribute knowledge that will be timely in making risk management decisions for the Libby site for the above reasons. Several of the proposed additional research projects in turn rely on the (to-be-developed) model

for translating the findings to information that would be practically useful for guiding risk management and policy decisions. These projects, while valuable for long-term research, may not be helpful for the short-term decision making needs at Libby. General thoughts on the usefulness of the NHEERL proposed research projects, as applied to Libby remedial cleanup decisions, are summarized below:

- *Model development to describe internal fiber burden.* As detailed above, this project would be useful for long-term research but unlikely to contribute significantly to upcoming risk management and policy decisions at Libby.
- *In vitro dissolution studies.* These studies will be useful for comparative purposes and would supply information needed for model development. However, it is already known that amphiboles in general dissolve much more slowly and thus are much more biopersistent than, for example, chrysotile asbestos. It is questionable whether new findings on dissolution rates specific to Libby amphibole would significantly alter cleanup decisions.
- *Animal intratracheal injection studies.* These studies would also be useful for comparative purposes with commercial asbestos materials and, in combination with current scientific knowledge, might allow determination of correction factors that could be used to apply risk models developed for commercial asbestos to Libby amphibole. To use the results directly in setting cleanup goals, however, findings on the basis of intratracheal dose would have to be correlated with and/or translated to an air concentration.
- *Animal inhalation studies.* These studies could provide data that would directly assist in setting cleanup levels, since exposure would be based on air levels.
- *Translocation studies.* These studies may be useful to answer basic research questions, although researchers have already shown translocation of amphiboles. It is questionable whether new findings on translocation specific to Libby amphibole would significantly alter cleanup decisions.
- *Studies on more-sensitive disease outcomes.* These studies may be useful for basic health intervention research. However, it is questionable whether findings would practically influence cleanup levels at Libby. Cleanup levels selected based on pulmonary endpoint-based risk may likely be near the limit of economic feasibility.

To summarize my recommendations:

- Immediate Libby risk management needs would best be served by focusing resources on animal inhalation disease outcome studies performed in a manner similar to animal experiments utilizing known commercial asbestos materials and previously reported in the literature.
- Animal intratracheal injection studies may also be useful to compare Libby amphibole effects with those reported in other studies.
- Other proposed projects are valuable long-term research projects to understand disease mechanisms and should be included as part of a separate long-term research plan.

John Wheeler's comments, 5/14/2007

Comments on NHEERL Research Project Plan

Concerns with developing an internal dose model for asbestos.

Obtaining the correct dose metric for evaluating toxicity from asbestos exposure has been an ongoing effort in the asbestos field for several decades. Developing a method to calculate internal dose of the biologically active forms of asbestos fibers (mineralogical and morphological) should enable the health/risk assessment field to better predict disease. It will also allow the collection of data that will help elucidate asbestos' mode and mechanism of action. The development will, however, require the examination of many model variables, will require a considerable research effort, and probably will not be timely enough to help in making clean-up decisions in Libby, Montana. Some concerns are listed below:

1) When the entire risk methodology for asbestos is considered, the one over-arching uncertainty is the exposure estimations from the 14 epidemiology studies that the methodology is based upon. We do not know if these studies over or under estimated exposure. Internal dose calculations will not improve these estimates and under certain conditions could worsen the dose-response estimates.
2) Trying to model the epidemiology studies has severe limitations. Not only is there wide uncertainty about the exposure estimates but there is little to no information available about the fiber size distributions. Internal distribution estimates would be meaningless without the proper fiber distribution data. The EPA's main concern with the Berman-Crump methodology was the surrogate data they employed to obtain fiber size distributions for the epidemiology studies. This proposal does not improve that limitation and is thus subject to the same criticism.
3) While there is evidence that longer fibers may increase toxicity, the role of fiber dimension is still undergoing considerable debate, especially for non-cancer endpoints. For developing a model, it could be assumed that fibers deposited in the bronchiole tree would lead to bronchogenic carcinomas and that fibers deposited in the alveolar region could lead to interstitial fibrosis. However, the understanding of deposition leading to mesothelioma is much less certain. While it appears that long fibers lodge in the lung and are therefore available to move to the mesothelium, microscopic examination of mesothelioma tissue shows short fibers. To clarify this picture for use in a deposition/clearance model, a major research effort would be required.
4) Clearance mechanisms are complex in asbestos analysis. A major hurdle would be that clearance would be dependent upon fiber size distributions that are unknown. In the case of lung cancer and asbestosis, clearance from the lung is likely beneficial and leads to decreased toxicity. Clearance in the case of mesothelioma and pleural disease could lead to less toxicity through macrophage clearance but to greater toxicity if cleared into the pleura. Even if the dose-response of this movement of fibers into the pleura was known or understood, the size fibers being cleared into the pleura that lead to disease is unknown.

Should the results of modeling internal dose result in an under- or over-estimation of toxicity in the same direction that the poor exposure data in the epidemiology has caused error, the results could magnify the error. At present there appears to be too many variables for which little data is available to make internal dose calculations without wide ranges of uncertainty.

At this time it appears that a better use of limited research funds would be to perform studies that accurately predict fiber size distributions in historical epidemiological studies. These values could be used both in the OSWER Interim Guidance and any future internal dose calculations.

(Received June 12, 2007)

NIEHS/NTP Comments on EPA/NHEERL Document: "Dosimetric and Toxicologic Assessment of Amphibole Fiber-Containing Material from Libby, Montana (April 30, 2007)"

The project outline is well-written and thorough. The points noted in the cover letter from L. Birnbaum requesting comments seem more appropriate to EPA internal reviewers. Thus, most of our comments focus on the relative utility and priority of the described projects, particularly as related to ensuring that the NTP can make use of the resulting analytical methods and toxicity data in developing a research program to characterize chronic toxicity/carcinogenicity of Libby amphibole (LA) and related mineral fibers in long-term inhalation studies. In summary, we view the materials characterization, dosimetry model development and thorough subchronic inhalation studies as the highest priority projects that will have the most impact on the design of NTP studies as well addressing current asbestos risk assessment uncertainties. We look forward to collaborating on implementation of this research program and offer assistance in specific study protocol development.

Test fiber samples
The use of different test materials for the *in vitro* and *in vivo* studies (i.e. 2000 vs 2007 sample collections) is a significant risk that should be carefully considered. At a minimum, the new sample should be first collected, characterized and compared to the old sample.

We realize that the identification and selection of test materials is difficult and unresolved but insufficient detail is provided to enable external reviewers to understand the scope of work and interrelatedness of research projects. What studies will be conducted on what materials?

How does the test material to be used compare to Libby environmental sampling? We recommend focusing more effort on demonstrating the relevance of materials that are prepared and selected for study to human exposure situations. This is work that EPA is well suited to do and is crucial for dosimetry model development and toxicity data interpretation.

Test materials should also be characterized for fiber type, size etc. after fractionation.

Comparisons to commercial asbestos (i.e. amosite) are important but the value of including SVFs, wollastonite as comparison is not clear.

Dosimetry model development
Is there an opportunity to use the existing or updated MPPD model to aid in designing/setting doses for the inhalation studies?

Dissolution assays
Dissolution studies should be conducted with LA first, as there will opportunities for methods refinement during the course of these studies. Though there is much data to compare to in the literature and the NHEERL database, it is reasonable to expect more difficulty conducting and interpreting these studies with a sample with mixed mineralogy/morphology.

Do the *in vitro* dissolution studies on LA and other fibers previously conducted by USGS supplant the need for new studies in any way?

In vitro toxicity assays
The *in vitro* studies with human primary cells will generate interesting data but their value as part of the overall plan is unclear. Will they be used to guide the design of the *in vivo* studies or used in dosimetry model development? If they are meant only to inform mechanism, how does each assay/endpoint relate to a particular adverse effect (e.g. cancer, asbestosis, autoimmune). Without parallel rodent cell culture studies, can they be related to the *in vivo* rodent data (e.g. inflammatory, oxidative stress markers)?

Fiber size- and shape-dependent disruption of macrophage function seems particularly worthwhile, as well as effects on signaling/gene expression in mesothelial cells.

Microarray analysis is semi-quantitative but not necessarily useful for deriving potency.

Comparative toxicology
Intranasal is not a preferred route of administration. Under what conditions would this be considered?

The proposed intratracheal studies are not providing much information that could not be obtained from inhalation studies of equivalent or shorter time course. Given the cost and time to set up the inhalation exposure facility for the LA subchronic study, there would be only incremental cost to do additional inhalation studies instead of intratracheal installation.

For evaluating the potential to cause autoimmune disease and mesothelioma, use of an appropriate animal model (e.g. Brown Norway rat) is the preferred approach. For evaluating genetic susceptibility, there should be clear (mechanistic) questions to address, not just selection of familiar models. What is the relationship of hypertension and atherosclerosis to asbestos induced health effects? How will these models shed light on susceptibility to mesothelioma?

Fiber translocation can be evaluated as part of the subchronic inhalation study. Why is a separate study proposed?

Inhalation toxicology
We recommend using Wistar Han rats. The NTP has recently switched to this animal model for all NTP studies (http://ntp.niehs.nih.gov/go/29502). This is important if the subchronic data is to be used in designing NTP chronic studies. The Wistar Han has been used in previous fiber studies (e.g. Bernstein et al. Inhal. Toxicol. 2005).

The proposed 5 day range-finding study may not be optimal for designing subchronic studies. Lung burden is critical for setting doses in subchronic studies and need some clearance time to get an idea of biopersistence. We recommend more dose groups (5). It might be useful to include amosite in the 5 day study as well.

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LIBBY AREA TECHNICAL ASSISTANCE GROUP, INC

May 29, 2007

Linda Birnbaum, Ph.D., D.A.B.T., A.T.S.
Director, Experimental Toxicology Division
EPA, ORD, NHEERL, Mail Drop B143-01
Research Triangle Park, NC 27711

Dear Dr. Birnbaum:

The Libby Area Technical Assistance Group (LATAG) is pleased to submit technical comments from our professional reviews to EPA, regarding ORD NHEERL Research Projects, proposed 30 April 2007, for "Dosimetric and Toxicologic Assessment of Amphibole Fiber-Containing Material from Libby, Montana."

Most of our comments were provided by the LATAG Technical Subcommittee, chaired by Dr. Brad Black (Lincoln County Health Officer) and including Dr. Gerry Henningsen as our TA (Technical Advisor). Dr. Black manages the Center for Asbestos Related Disease (CARD, www.libbyasbestos.org) where he and Dr. Alan Whitehouse (MD pulmonologist from Spokane, WA), along with staff, treat clinically diseased patients and screen persons who were exposed to Libby Amphibole (LA) asbestos. Dr. Henningsen has expertise in Superfund environmental risk assessment, as a past senior toxicologist at EPA R8 for 10 years, and in occupational and comparative toxicology research (working at WPAFB for 3 years, and at NIOSH for 5 years, where he also served as a NTP Chemical Manager).

Dr. Black and Dr. Henningsen conferred with several of their scientific colleagues who are well known and respected national experts in asbestos toxicology. Their professional opinions were consistent and unanimous by concluding that, while NHEERL lab and staff have excellent scientific capabilities, the proposed research projects will mostly fail to provide useful applied data that are considered most important and urgently needed by EPA R8 and Libby to assess quantitative risks of disease from exposures to LA asbestos.

The LATAG agrees with our Technical Subcommittee's reviews, and requests that EPA start over with their risk-investigation proposal. We recommend that EPA create a small panel of expert scientists, facilitated by R8 toxicologists, that includes our Technical Sub-committee, R8 toxicologists, ORD scientists, some stakeholder scientists, and other expert consultants as needed. We believe this panel should effectively help EPA to clearly define their risk management goals, prioritize study objectives, review study proposals / results / reports, and communicate risks of LA asbestos, from results of site investigations that fill critical data-gaps and reduce uncertainties of risks. These results are required by R8 to provide protective remedial cleanups at Libby. We need rapid and practical risk-based answers from EPA, since many of our citizens are victims who continue to die and suffer from progressive diseases due to LA exposures. We strongly urge EPA to use this local panel of biomedical experts to help design and prioritize better site-specific studies of LA asbestos, and we request approval to select up to 6 expert scientists to serve on this panel.

Thank you for considering our technical comments, which are intended to offer EPA some constructive suggestions to improve results and success at Libby. We invite you, and also welcome your EPA research colleagues, to visit our wonderful community very soon.

Sincerely,

Mike Noble
Chair, LATAG

Attachments: Technical Review Comments from the LATAG to EPA on LA research
Copies to: Senator Max Baucus, Senator Jon Testor, EPA R8, EPA Superfund HQ

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Ref: Technical Review by TA of EPA ORD/NHEERL Research Proposals, 30 Apr 2007: Dosimetric and Toxicologic Assessment of Amphibole Fiber-Containing Material from Libby, Montana

Dear LATAG:

Background. I professionally reviewed, as Technical Advisor (TA) for Libby Area Technical Assistance Group (LATAG), the subject document and supporting materials. This draft proposal plans to conduct new research studies for dual uses by EPA, both for R8's need to improve risk assessments of exposures to Libby Amphibole (LA) asbestos and for broader concerns about amphibole risks. Draft documents were provided by Paul Peronard, R8 lead-RPM (remedial project manager) for the Libby NPL (national priority list; i.e., Superfund) site, forwarded from Dr. Stephen Gavett at NHEERL (National Health and Environmental Effects Research Laboratory) in Research Triangle Park (RTP), NC, which is part of the EPA Office of Research and Development (ORD) program. For information to readers of this review, I included: 1) Paul's transmittal email, containing Dr. Gavett's email message and attachments for peer-review, 2) cover letter by Dr. Linda Birnbaum, Director, Experimental Toxicology Division of NHEERL, and 3) EPA's draft revised proposal, dated 30 Apr 2007, that contains my comments. Where possible, I tried to provide constructive suggestions for EPA to consider in revisions or for responding to comments.

Conclusions. After conducting my review, I conferred with several scientific colleagues who are well known and respected as national experts in asbestos toxicology. Their professional opinions were consistent with mine; whereby I conclude (while acknowledging excellent scientific capabilities and reputation of NHEERL) that the proposed research projects are misdirected. NHEERL missed the main purposes of supporting R8 for conducting the most urgent and practical toxicological studies of LA, that are critically needed to supply essential site-specific data for quantitative risk assessment at Libby. Current lab proposals will certainly fall short of the goals and needs of R8 and Libby to obtain usable data (derived from applied research studies and prioritized site-specific investigations), which are urgently required.

Rationale. In other words, R8 and Libby do not need so much basic research that is currently proposed by NHEERL, but R8 mostly needs realistic "testing" of the toxicity on LA asbestos for prioritized risk-based data-gaps; e.g., determine the "relative toxic potency" of representative LA asbestos fibers, compared to standardized samples of chrysotile and amphibole fibers. While the mostly basic research that is outlined in the current draft proposal may be interesting, it has little practical use for helping NPL sites that are contaminated with amphibole asbestos fibers. Applicability and priority of the proposed lab studies are low, for purposes of assessing quantitative risks at Libby or helping R8 RPMs to make strong remedial decisions that protect health at Libby per EPA's criteria. Despite my negative review about NHEERL's proposal, which is not intended to be perceived as a critical personal attack, R8 (with help

from NHEERL and other scientists) can use this review and others' comments as an opportunity to provide new solutions to conduct more useful studies that target the most critical risk needs at Libby.

Recommendations. I suggest that EPA should essentially start this research project anew, and adopt a practical site-specific approach for proposing applied lab research and risk-based site investigations of LA asbestos at Libby. EPA should create an small, effective panel of outside expert scientists who can independently and objectively review EPA's proposed research; which is facilitated locally by R8 toxicologists, and includes the LATAG Technical Subcommittee, R8 scientists, key ORD scientists, and other stakeholder scientists as needed. Expert science panels were previously suggested for use at Libby, since they succeeded (efficiently and effectively) at many R8 NPL sites with complex science issues that initially seemed foreboding; e.g., California Gulch, Summitville, Kennecott, Rocky Mountain Arsenal, Smuggler Mountain, Montecello, Butte, Anaconda, etc. This panel should be tasked to objectively apply best available science to help EPA clearly define or refine risk management goals, prioritize study objectives, review study proposals/results/reports, and communicate risks of LA asbestos, with focus on site investigations that fill critical data-gaps and reduce uncertainties of risks. For equitable scientific representation of Libby, the LATAG should name at least 6 expert scientist members to this panel.

Approach. Libby needs rapid and practical risk-based answers from EPA researchers and their contract laboratories, since many human victims continue to die and suffer from progressive diseases that result from over-exposures to LA asbestos. EPA is therefore strongly urged to promptly employ a local panel of biomedical experts to help enhance study designs for prioritized site-specific investigations that will generate sound scientific data for applied use in risk assessments of LA. EPA should focus only on the most vital studies first for R8 use at Libby, and plan to conduct multiple categories of research in tiers or phases as needed to **reduce uncertainties and fill remaining important data-gaps**. Main **categories** of site studies that are needed with **high priority** at Libby include:

1. **epidemiology and clinically derived exposure data** on major pathways of exposure to LA, surveys of highly exposed subpopulations at risk of ARD (Plummer School children, Libby Dam)
2. **toxicology** of LA, focused on **relative potency** vs chrystotile and amphibole standards, causes of severe **inflammation and fibrosis** in target tissues, susceptibility factors, co-toxicants if any, define **proximate toxicant** in the mixture of amphiboles (geophysically and chemically)
3. sampling and analyses of **cumulative exposures** to airborne LA and other potential sources at Libby, and defining **geographical extents of contamination and nature** of LA fibers present
4. analytical **methods refinement or new method developmen**t (e.g., SEM, morphometrics) to detect and quantify **risk-based trace levels** of short fibers and fragments, typical structures, and long fibers at concentrations far below current MDLs of about 0.0002 f/cc for risk needs
5. **computational support** to organize data into **usable** forms, and to **model** (if valid for fibers and calibrated) exposure and dose responses that include estimates of **variability and uncertainty**
6. **ecological effects** of sensitive receptors, including endangered species in the watershed

These results are foremost required by R8 before EPA can propose or provide protective remedial cleanups at Libby, helping to assure citizens of long-term health protection for their families, and

defending actions against critics, scientific peers, and legal challenges in court (Superfund risk-based data help to justify cost-recovery of remedial expenses). However, few of the above risk-related needs are adequately addressed in the research proposed by NHEERL. The panel of outside expert scientists should be able to help R8 fully plan and justify priorities of risk studies needed to fill major data-gaps and to reduce uncertainties at Libby.

Communications. Coincidentally, the R8 Libby site team is took the initiative to engage in discussions about risk-related investigations with the LATAG Technical Subcommittee during May 21-22, 2007. This meeting improved mutual understanding of differing perspectives about the needs for various types and amounts of risk-based data at Libby; and the group explored ways to help make better use of existing scientific expertise to help generate essential site data for application in quantitative risk assessments. For completeness of information, a copy of the draft agenda is attached for the upcoming meeting between R8 and the LATAG representatives. A synopsis of technical feedback, provided to R8 by me as TA, is paraphrased at the end of Attachment 2; much of which applies to the NHEERL research proposal.

Major technical review comments, per Cover Letter charges.

1. Purpose of the research is stated as assessing dosimetric and toxicologic effects of amphibole fiber-containing vermiculite, per the IG's recommendation to conduct research on the toxicity of Libby amphibole asbestos; however, I **do not foresee much usable data** on toxicological dose-response coming from these studies for risk assessment.

2. Action Plan from the Jan 2007 meeting evolved into this draft NHEERL proposal, with ~$3M extended over 3 years to conduct research; however, there is **no structured research strategy** that clearly defines the problem statement, study objectives, relative priorities, or anticipated research data that will have specified uses for helping to assess risks at Libby. This information needs to be clearly determined prior to the start of any research by EPA, which should clearly address the top risk needs at the site by filling critical data-gaps and reducing major uncertainties.

    a. The proposal is supposed to address key uncertainties of risks, but these **uncertainties are not clearly described or defined with prioritized needs**; so it is difficult to imagine how the research will actually reduce key uncertainties for risk assessments at Libby.

    b. EPA has broader applications for the studies, with 2 other asbestos samples to be tested; however, **rationale for selecting the other two samples is vague** and weakly supported. Research on LA amphibole should have broader applications, but these need to be clearly defined for best prospects of meeting R8 risk management goals and study objectives.

    c. Five proposed research areas include:

        i. In-vitro dissolution; this may be interesting but has **low priority**, since it will be difficult to determine fate and translocation of Libby amphibole in situ, so these studies should be relegated with lower priorities to later times.

        ii. In vitro toxicity; cellular responses are again interesting but are also difficult to

extrapolate and predict human responses, so the study should also be relegated with **lower priorities** to later times.

iii. Intratracheal installation of LA in rodents – just **use Wistar rats** and/or non-human primates, since **mice are not needed** and are questionable models for asbestosis and human lung cancer; **other routes of administration** may be as informative about the pathogenesis and relative potency of Libby amphibole, such as **intrapleural and intraperitoneal instillations** of known quantities, sizes, and chemistries of fibers.

iv. Subchronic inhalation in rats – use **Wistar rats**, and not the F344 strain, since Wistars have less confounding cancer and other diseases; what is the purpose for only exposing rats to Libby amphibole for 90 **"partial"** days (**only 15 days of continuous exposure divided over 90days!**), when the spectrum of asbestos related diseases (ARD) are thought to occur after chronic "**continuous**" (likely intermittent excursions) exposure with varying latency times; thus, subchronic exposure may **miss critical disease endpoints of relevance** to Libby amphibole.

v. Dosimetric models of above results; the models can be used to **estimate various endpoints** of disease, but they **must be validated for their predictability** and **calibrated for use at specific sites** with unique conditions. Therefore, modeling of risks from estimated dosimetry inputs and kinetic assumptions is highly uncertain and should be relegated with **lower priority** to later times.

3. The proposal contains an outline, without study designs or detailed protocols; therefore, along with **missing goals and objectives with highest priorities** for use to assess risks at Libby, this proposal is **not convincing** for its ability to provide the quality and quantity of site information that is expected by the instigating parties and is critically needed by R8 for risk assessment. Internal review of protocols is only planned by EPA; but these **protocols should also have external expert reviews**, after **major revisions** are made to propose studies that more strongly and directly address prioritized risk issues at Libby.

4. Overall direction of proposals: this reviewer is **not confident that the direction and research products will be useful for risk assessment** at Libby.

   a. *Do proposed in vitro, in vivo and dosimetry studies address the risk appropriately?* **No**, they do not, and **fall short** of meeting the most important risk needs at Libby.

   b. *Are products useful to R8?* **Doubtful**; since little practical data appear to be able to be generated for risk assessment by the current proposed studies, which are incomplete as well as misdirected for R8 risk assessment purposes.

   c. *Improvements for achieving goals*? EPA needs to **fully characterize samples** of Libby amphibole that are representative of typical in ranges of exposures to this asbestos fiber mixture. Unless EPA **first establishes a suitably representative and standardized test**

material of Libby amphibole and comparative asbestos fibers, and research studies will be **compromised** and results may be **limited or useless** for risk assessment.

d. *Funding and timelines reasonable, for ROD in 2010?* **No**. From experience at many other Superfund sites, we estimated a **minimum of at least triple that amount (i.e., $11M) within 3 to 5 years** to address the major (tier 1) prioritized risk needs at Libby.

e. *Support R8 risk assessment of naturally occurring asbestos?* **No**! Where do you get the **misunderstanding** that Libby amphibole is naturally occurring asbestos in that community's environment? Most of the contamination is from distribution and dissemination of processed vermiculite ores into expanded product and insulation materials that was spread throughout Libby during the past decades with current cycles of re-contamination.

Specific technical review comments. Edits are provided in the following document. Obviously, this reviewer strongly believes, from a basis of experience with Superfund sites, that this proposal needs to be completely re-thought with a meaningful focus on realistic and prioritized risk needs to produce results that fill critical data-gaps and reduce uncertainties in risks. Therefore, minimal comments are made for the current draft proposal, since most of those studies are inadequate as written to fulfill risk needs at Libby. Convening an expert panel of scientists, that is small enough to work effectively and efficiently, that are managed locally byR8 will help to propose the major set of critical studies that are needed at Libby with specific study objectives to meet risk management goals. EPA NHEERL can and should play an important role as participants, but not take the lead in managing and directing this panel. The expert panels of scientist with appropriate experience and knowledge have proven to be successful many times at the most complex superfund sites in R8 EPA; such a panel is strongly recommended for use at Libby.

Please contact me if the LATAG needs additional information on my TA review of this proposal. Thank you for the opportunity to professionally serve the scientific needs of the LATAG and Libby residents.

Sincerely yours,

Gerry M Henningsen

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Attachments:
1. Final Document sent to reviewers this morning, 30Apr 2007 email from Paul Peronard, EPA R8
2. EPA R8 draft agenda for meeting with LATAG representatives on 21-22 May 2007
3. Libby Plans int rev, 30 Apr 2007 cover letter from Dr. Linda Birnbaum, EPA/ORD/NHEERL
4. NHEERL planned LA studies.doc, 30 Apr 2007, with edits inserted by TA for LATAG

Copies to: draft comments to EPA R8, attn: Paul Peronard, Jim Luey, and Chris Weis; Linda Birnbaum