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WORKSHOP REPORT

Chronic Inhalation Toxicity and Carcinogenicity Testing of Respirable Fibrous Particles[1]

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On May 8–10, 1995, a workshop on chronic inhalation toxicity and carcinogenicity testing of respirable fibrous particles was held in Chapel Hill, North Carolina. The workshop was sponsored by the Office of Pollution Prevention and Toxics, U.S. Environmental Protection Agency (EPA), in collaboration with the National Institute of Environmental Health Sciences (NIEHS), the National Institute for Occupational Safety and Health (NIOSH), and the Occupational Safety and Health Administration (OSHA). The goal of the workshop was to obtain input from the scientific community on a number of issues related to fiber testing. Major issues for discussion were: (i) the optimal design and conduct of studies of the health effects of chronic inhalation exposure of animals to fibers; (ii) preliminary studies which would be useful guides in designing the chronic exposure study; (iii) mechanistic studies which would be important adjuncts to the chronic exposure study to enable better interpretation of study results and extrapolation of potential effects in exposed humans; and (iv) available screening tests which can be used to develop a minimum data set for (a) making decisions about the potential health hazard of the fibers and (b) prioritizing the need for further testing in a chronic inhalation study. After extensive discussion and debate of the workshop issues, the general consensus of the expert panel is that chronic inhalation studies of fibers in the rat are the most appropriate tests for predicting inhalation hazard and risk of fibers to humans. A number of guidances specific for the design and conduct of prechronic and chronic inhalation studies of fibers in rodents were recommended. For instance, it was recommended that along with other information (decrease in body weight, systemic toxicity, etc.), data should be obtained on lung burdens and bronchoalveolar lavage fluid analysis to assist in establishing the chronic exposure levels. Lung burden data are also important for quantifying aspects of risk assessment related to dosimetric adjustments before extrapolation. Although mechanistic studies are not recommended as part of the standard chronic inhalation studies, the expert panel stressed the need for obtaining mechanistic information as far as possible during the course of subchronic or chronic inhalation studies. At present, no single assay and battery of short-term assays can predict the outcome of a chronic inhalation bioassay with respect to carcinogenic effects. Meanwhile, several short-term *in vitro* and *in vivo* studies that may be useful to assess the relative potential of fibrous substances to cause lung toxicity/carcinogenicity have been identified.

INTRODUCTION

An important task for environmental protection is to identify and subsequently to prevent, eliminate, or mitigate the risks to human health and the environment posed by toxic substances. Natural and synthetic fibers are one group of substances that have been identified to be of potential concern. Many of these fibers have wide industrial and commercial applications, and for some there are limited, inconclusive, or virtually no

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information about their health effects and/or exposure to workers, consumers, and the general public. As a result, the U.S. Environmental Protection Agency (EPA) has added a "respirable fibers" category as priority substance for health effects and exposure testing to obtain the necessary data to evaluate the extent and magnitude of health risks to exposed individuals and populations. This would then allow the Agency to determine whether there is a basis for any risk reduction measures.

The health endpoints of concern for respirable fibers are the potential development of respiratory diseases including cancer from chronic inhalation exposure. In humans, the inhalation of asbestos and erionite fibers has been associated with the development of nonmalignant and malignant diseases, primarily of the lung, pleura, and peritoneum. The mechanisms by which these fibers induce diseases in humans are not clearly understood. It is generally believed, however, that the potential toxicity and carcinogenicity of a given fiber type appear to be dependent upon the respirability and biopersistence of the particle, i.e., the ability of the fiber to enter the respiratory tract and penetrate into the alveolar region of the lung, and to be retained in the lung.

EPA recognizes that the current health effects test guidelines for chronic inhalation toxicity and/or carcinogenicity studies are not sufficiently specific for the testing of fibrous substances. Thus, there is a need for EPA to develop standardized health effects test guidelines for fibrous substances that can be used by EPA in future rulemaking, negotiated enforceable consent agreement, or voluntary action to obtain the necessary toxicologic information for risk assessment. However, at present, there is no general agreement upon test protocols for chronic inhalation toxicity and carcinogenicity testing of fibers for regulatory purposes. It is, therefore, important for the Agency to obtain input from the scientific community on a number of issues related to fiber testing prior to the development of standardized health effects test guidelines for fibrous substances.

On May 8–10, 1995, a workshop on chronic inhalation toxicity and carcinogenicity testing of respirable fibrous particles was held at Chapel Hill, North Carolina. The workshop was sponsored by the Office of Pollution Prevention and Toxics, EPA, in collaboration with the National Institute of Environmental Health Sciences (NIEHS), the National Institute for Occupational Safety and Health (NIOSH), and the Occupational Safety and Health Administration (OSHA). The Steering Committee (authors of the present paper), which was responsible for the planning of the workshop, was composed of representatives of each of these health protection/regulatory government agencies. The workshop was conducted by Research Evaluation Associates, Inc., an EPA contractor who was responsible for convening the expert panel which was composed of 19 international expert scientists in inhalation toxicology. The expert panel (see Appendix) reviewed, evaluated, and commented on the scientific issues of the workshop. Dr. Günter Oberdörster of the University of Rochester was the workshop chair and a number of other members of the expert panel served as session chairs and rapporteurs. More than 60 participants from the government, industry, academia, and the general public attended this workshop. The workshop agenda, list of participants, and the full account of the panel discussions have been published in an EPA report (USEPA, 1996). The present report summarizes the workshop issues identified in an issue paper prepared by the Steering Committee and the panel's conclusions and recommendations prepared by the rapporteurs of the workshop.

WORKSHOP OBJECTIVE

The objective of the workshop was to obtain guidance from the scientific community on a number of issues related to chronic inhalation toxicity/carcinogenicity testing of fibrous substances. Major issues involved include the following:

(1) the optimal design and conduct of studies of the health effects of chronic inhalation exposure of animals to fibers;

(2) preliminary studies which would be useful guides in designing the chronic exposure study;

(3) mechanistic studies which would be important adjuncts to the chronic exposure study to enable better interpretation of study results and extrapolation of potential effects in exposed humans; and

(4) available screening tests which can be used to develop a minimum data set for (a) making decisions about the potential health hazard of the fibers and (b) prioritizing the need for further testing in a chronic inhalation study.

The expert panel was asked to review a background document (Oberdörster, 1995; in Appendix IV, USEPA, 1996) which provides an overview of major issues related to toxicologic testing of respirable fibrous particles and an issue paper (Appendix III, USEPA, 1996) which presents the issues for workshop discussions.

WORKSHOP ISSUES AND PANEL RECOMMENDATIONS

The workshop issues were grouped into seven major topics for discussion purposes.

I. Inhalation Exposures: Materials and Methods

I.1. Definition of Fibers

Fibers are generally defined as elongated particles with a length-to-diameter ratio (i.e., aspect ratio) equal

to or greater than 3 to 1. This definition is presumed to include particles with varying shapes such as rod-like, curly, or acicular (needle-like) shapes, and having different structural units commonly referred to as fibers, fibrils, or whiskers.

Fibers of most concern to humans appear to be confined to the thoracic particulate fraction. For instance, it has been shown that humans exposed to asbestos fibers develop lung tumors both in the conducting airways and in the peripheral regions. In contrast, lung tumors are induced in the peripheral regions in rats after inhalation of fibers, indicating that respirable fibers are the most hazardous ones for rats. Furthermore, longer human respirable fibers which are hazardous to humans may not be respirable by the rat. On the other hand, because of the smaller size of rat phagocytic cells, shorter fibers may be carcinogenic in rats but may be readily cleared in humans.

Question 1: Is an aspect ratio of equal to or greater than 3:1 an acceptable definition of a fiber? If not, how should the definition be modified to encompass the varying range of sizes and shapes of naturally occurring and synthetic fibrous substances?

Question 2: Since fibers of potential health concern to humans are those that are deposited in the entire lung, not just in the gas exchange region, a more correct term would be thoracic fibers rather than respirable fibers. What would be appropriate definitions of human "thoracic" fibers and "respirable" fibers? What would be a suitable definition of rat "respirable" fibers?

Conclusions and recommendations: A fiber is defined as a particle having an aspect ratio of at least 3:1 (length:diameter) and being structurally continuous.

Respirability should be defined on the basis of experimental data, rather than calculated data. The term "respirable fiber" should always be used with a species modifier, such as "human-respirable" or "rat-respirable." "Respirable" means that the particle in question can penetrate to the alveolar region upon inhalation. A "rat-respirable fiber" is defined as a fiber having an aerodynamic diameter of less than 3 μm. "Thoracic" fibers were defined by the participants only in general terms as those fibers penetrating to the conducting airways upon inhalation.

I.2. Selection Criteria for Suitable Test Materials

As discussed above, there are considerable differences in fiber inhalability and respirability between humans and laboratory rodents. This observation raises several questions with respect to the choices of fiber samples to be tested, recognizing the inherent limitations of using rodent species as surrogates of humans in inhalation studies, and the need for optimizing the study conditions while still being able to obtain pertinent toxicologic information for extrapolations to humans.

Question 1: For a given fiber type, should inhalation studies be performed using samples with the greatest potential for pathogenic effects (e.g., long, thin fibers)?

Question 2: Should fiber samples for testing be prepared so that they are rodent-respirable, or should they represent a human-respirable sample (or fibers conforming to a human thoracic particle definition)?

Question 3: Should the test fibers reflect what are actually present at the workplace and/or nonoccupational environments?

Question 4: In the case of new fibers, how should the test materials be selected?

Conclusions and recommendations. It was suggested that to maximize sensitivity of animal inhalation exposure studies for determining health effects of fibers, the test material should consist of rat-respirable fibers and should be enriched with the most potent human-respirable fraction (i.e., long, thin fibers); therefore, rodent inhalation exposure studies should use an exposure aerosol that is, as far as is technically feasible, enriched with the following fiber size fractions: rat-respirable fibers with aspect ratio of at least 3:1 and aerodynamic diameter less than 3 μm, and human-respirable fibers with lengths of at least 20 μm or fibers with high aspect ratios. The fraction of long fibers ($>$20 μm) should be specified; 10 to 20% enrichment would seem appropriate, but not enough information is available on which to base a specified percentage. The aerosolized fibers should be discharged to Boltzmann equilibrium before being delivered to the test species.

If the study results are negative and it can be shown that fiber mass loading and fiber size distribution in the lung are not sufficient, then the fiber would be considered to not have been adequately tested.

I.3. Characterization of Test Fibers

There is considerable evidence to suggest the importance of fiber characteristics in relation to disease outcomes. Thus, it is desirable to obtain data on a number of the physical and chemical properties of the particles. These data will also enable the investigator to make some preliminary estimates of the lung burden of the material at a given exposure concentration, the behavior of the particle in the lung, and to some extent, its expected toxicity.

Question 1: At a minimum, what aspects of the test samples need to be characterized (e.g., fiber morphology, dimension, size distribution, aerodynamic diameter, chemistry, density, solubility, surface characteristics, the ability of a fiber to split longitudinally or cross-sectionally)?

Question 2: Are there any specific analytical methods that should be required to be used to characterize certain chemical and physical properties of bulk materials or individual fibers present in the aerosol and lung tissues?

Question 3: Are the available methods for the measurement of fiber size distributions considered adequate? Are there any new and improved methods that can be used for measuring fiber size distributions?

Conclusions and recommendations. The complete bivariate length and diameter distribution should be determined in the aerosol and in the lung via electron microscopy. This bivariate distribution should include the nonfibrous particles present in the aerosol. For nonfibrous particles, "length" and "diameter" can be determined as the "longest length" and "narrowest dimension." The aerodynamic size distribution should be determined; a cascade impactor can be used for this purpose. The World Health Organization (WHO) counting rules and sizing rules should be used and results should be evaluated statistically to assure sufficient sensitivity. Interlaboratory validation should be provided for all counting and sizing methods. In addition, it is recommended that sizing techniques be used that permit returning to the same fields and fibers in the event it is necessary to confirm counting. Routine monitoring to control the day-to-day aerosol concentration can be performed using phase-contrast optical microscopy (PCOM) and gravimetric techniques.

I.4. Exposure Conditions and Methods

There are certain disadvantages associated with either whole-body nose-only exposure: greater stress of the animals with nose-only method and ingestion from grooming with whole-body method. Both methods are considered acceptable by EPA and other regulatory authorities as appropriate methods for inhalation testing of chemical substances. Regardless of the exposure method used, fiber samples need to be aerosolized in such a way that they are evenly distributed in the chamber atmosphere and that there is adequate sampling to verify the integrity of the aerosolized fibers and that constant fiber concentrations are maintained throughout the exposure period.

Question 1: Should there be a requirement for the use of any particular method for generating fibrous aerosols?

Question 2: Are both methods of exposure (whole-body and nose-only exposures) acceptable? Is there a preferred method that should be recommended?

Question 3: How often should exposure atmosphere be monitored with regard to fiber number and mass concentration, size distribution, and chemical analysis?

Conclusions and recommendations. The test guidelines should not specify a particular aerosol generation system, but should require that the exposure system be validated by the investigator (e.g., with respect to airborne fiber size distribution and the target dose in the lung). It should be demonstrated that the generation system does not contaminate the fibers.

Either nose-only or whole-body exposure can be used. The target exposure concentrations should be measured regularly during the course of the study by electron microscopy (fiber number and bivariate size distribution) to confirm the dose delivered to the animals. The frequency of exposure atmosphere monitoring should be daily for mass concentration, weekly for fiber concentration and bivariate size distribution, every 3 months for chemical analysis.

II. Study Design for Chronic Inhalation Studies

II.1. Animal Species/Strain/Sex Selection

EPA's current test guidelines for oncogenicity require that a compound of unknown activity shall be tested on two mammalian species via oral, inhalation, or dermal route of exposure. Rats and mice of both sexes are the species of choice without specifying more precisely any specific strains, except that commonly used laboratory strains shall be employed. Justification when selecting other species must be provided. Rats and mice are the species of choice mainly because of their relatively short life spans, their widespread use in toxicological studies, their susceptibility to tumor induction, and the limited cost of their maintenance. However, for combined chronic toxicity and oncogenicity study, the rat is the species of choice.

Inhalation studies with asbestos fibers in rats have been demonstrated to be appropriate experimental models for the identification of asbestos-induced human diseases, primarily fibrosis and cancer of the lung. The low mesothelioma rate induced in rats via inhalation, compared with the rate of crocidolite-induced mesotheliomas in humans, indicates that the rat inhalation model may not be adequately sensitive to identify the potential ability of fibers of unknown activity to induce mesothelioma in humans. Exceptions occur when the fiber in question is expected to be a potent mesothelioma inducer such as erionite fiber. Since induction of mesothelioma is also a health endpoint of concern, testing in a second rodent species may be necessary to ensure that all potential health effects would be properly identified.

On the other hand, questions have been raised about the validity and utility of using either the mouse or the hamster as the second species for carcinogenicity testing of fibers. The concern is that there have been fewer studies using mice and hamsters with asbestos fibers and, although many of these studies have been considered limited (e.g., using short asbestos fibers, short duration of exposure), results obtained to date seem to indicate that they may not be suitable animal models for predicting asbestos fiber-induced diseases. The mouse generally does not respond to tumor induction by asbestos fibers via inhalation. In the case of the hamster, this species appears to be more sensitive than the rat with respect to fiber-induced mesothelioma, but

less sensitive to the induction of lung tumors and fibrosis than the rat.

Question 1: Is it necessary to test fibers of unknown activity in a second animal species? If yes, what would be an appropriate second species (e.g., Syrian Golden hamster, Chinese hamster)? If not, what are the scientific reasons? Are there any circumstances that warrant testing in a second species?

Question 2: Do different animal strains respond with different sensitivities to fibrous particles? If yes, what is the most appropriate strain of rodent species to be used?

Question 3: Should both sexes of the animal be used? If not, which sex is more suitable, and why?

Conclusions and recommendations. Given the present limited knowledge of the effects of fibers in species other than the rat, testing of fibers in a second animal species is not strongly recommended; however, investigators should be encouraged to investigate health effects of fibers in another species, particularly the hamster. In the future, transgenic animals may prove useful for testing.

Although it was acknowledged that rat strains clearly differ in suitability for inhalation exposure studies with fibers, the panel could not agree on whether to recommend a particular strain to use (or to avoid), but proposed a set of criteria for choosing a strain. It was agreed that criteria for a suitable strain include (1) a low background rate of neoplasia, (2) a low background rate of pulmonary disease, (3) longevity, and (4) a history of laboratory use.

It was agreed that whether to use one or both sexes should be left to the investigator, and that if one sex is used, the choice should depend on factors related to the strain, test material, and endpoint studied. However, if cost was not a factor, testing in both sexes should be encouraged because data on sex differences in response to inhalation exposure to fibers are limited.

II.2. Selection of Exposure Concentrations

EPA's current test guidelines for oncogenicity require at least three exposure concentrations and a sham-exposed (filtered air only) control group. The highest concentration should elicit signs of minimal toxicity without substantially altering the normal life span other than tumor formation. The lowest exposure concentration should not induce any indications of toxicity and the intermediate concentration(s) should be established in a mid-range between high and low concentrations. There is no specific guidance on how to select aerosol concentrations for inhalation studies of particulate matter.

For combined chronic toxicity and oncogenicity studies, EPA's guidelines require the use of a high concentration treated and control satellite group designed to evaluate pathology other than neoplasia. The highest concentration for satellite animals should be chosen to produce frank toxicity, but not excessive lethality.

Recently, a number of criteria (e.g., effect on lung clearance and pulmonary function, chronic inflammatory responses, cell proliferation, histopathological changes) have been proposed to define the highest fiber concentrations to be tested in a chronic study, also known as the maximum aerosol concentration or MAC.

Question 1: What criteria can be used to determine the maximum aerosol concentration (MAC) in inhalation studies of fibrous particulates and to judge whether a MAC has been reached or exceeded?

Question 2: What preliminary studies would be useful and important for setting appropriate exposure concentrations (e.g., 90-day and/or shorter-term inhalation studies, *in vitro* solubility, *in vivo* biopersistence studies)?

Question 3: The National Toxicology Program (NTP) generally employs an upper limit exposure concentration of 100 mg/m^3 for relatively insoluble nonfibrous particles of low toxicity. In view of potential particle "overload," should a practical upper limit concentration also be set for fibrous particles?

Conclusions and recommendations. A practical upper limit concentration was not endorsed since it would depend on fiber type, and no one number could be determined that applies to all fibers. The MAC should be based on the total number of inhaled particles (fibers and nonfibrous particles combined). The MAC should be considered based on a combination of the following parameters determined in a 90-day subchronic inhalation study: altered alveolar macrophage-mediated particle clearance rate, fiber lung burden normalized to exposure concentration, cell proliferation, histopathology, inflammation (quantitatively determined as percentage increase in polymorphonuclear leukocytes [PMNs] in lung lavage samples) and lung weight. It was suggested that an appropriate lung burden of critical fibers (long and thin) should be achieved, but no number was suggested. These parameters should be considered together, rather than individually, in an attempt to define a maximum tolerated dose (MTD) for the chronic study. The MTD is the lung dose achieved with the MAC. For the chronic study, three exposure concentrations should be used; the high exposure concentration and resulting lung dose should show significant effects in the above parameters (MTD), and the lower doses should be appropriately spaced and be selected based on results from the 90-day study and from previous studies with the particular fiber. Ancillary studies should be conducted to determine *in vitro* solubility and *in vivo* biopersistence.

II.3. Exposure Regimen and Observation Period

EPA's current test guidelines require that the animals are exposed to the test substance for 6 hr per day, 5 days per week over a period of at least 24 months for

rats, and 18 months for mice and hamsters. Termination of the study should be at 24 months and not longer than 30 months for rats, and at 18 months and not longer than 24 months for mice and hamsters. However, termination of the study is acceptable when the number of survivors of the lower exposure groups or of controls reaches 25%.

Experience with asbestos fibers indicates that fiber-induced lung tumors or mesothelioma in rats occur at relatively advanced age. Thus, it would be desirable to allow the animals to live out their life span after the 2-year exposure is completed. On the other hand, there are disadvantages of a lifetime study. These include the high mortality rate in rats over 2 years of age, and the high incidence of age-related spontaneous nonneoplastic and neoplastic lesions which would make interpretation of study findings difficult.

Question 1: Is the exposure regimen as specified in EPA's guidelines appropriate for the testing of fibers?

Question 2: Is it necessary to recommend when final sacrifice be carried out? If so, when would it be?

Conclusions and recommendations. The chronic inhalation exposure study with fibers should be a lifetime study with exposure terminated at 24 months in rats and the study terminated when survival of the control group reaches 20%. Due to the shorter life span of hamsters, their exposure duration could be shorter, based upon survival/lifetime expectancy.

II.4. Numbers of Animals and Interim Sacrifices

EPA's current test guidelines require that at least 100 animals (50 males and 50 females) are to be used for each exposed and sham-exposed control groups. Satellite exposed and control groups consisting of 20 males and 20 females are to be used in the combined chronic toxicity and oncogenicity study. Additional animals are used if interim sacrifices are planned. However, this is optional.

Question: Should interim sacrifices be recommended for the testing of fibers? If yes, what would be an appropriate interim sacrifice schedule and design (e.g., number of animals per group, duration of exposure and recovery period)?

Conclusions and recommendations. Interim sacrifices are essential and should be made at 3, 6, 12, 18, and 24 months in rats. The endpoints evaluated at these times should be the same as in the subchronic study. Since hamsters do not live as long as rats, a study of 24 months exposure may not be possible in this species. Lung clearance of particles in live animals also should be measured at set intervals. Investigators should be encouraged also to follow recovery in animals exposed for shorter periods and sacrificed at the same intervals. The number of animals will depend on the specific study design.

II.5. Use of Positive Control

The use of a positive control is not required in EPA's current test guidelines for chronic toxicity and oncogenicity testing. However, in view of the complexity of conducting an inhalation study with fibrous particles, it may be useful to consider including a group of positive controls to validate the reliability of the testing system. Asbestos fibers are most often used as a positive control, but exposure-dose–response relationships have not yet been established for any types of asbestos fibers. Moreover, standardized UICC reference materials (e.g., UICC crocidolite) have been considered not suitable because of their short fiber length.

Question 1: Should a positive control be included in the chronic inhalation study with fibers? If not, why?

Question 2: If yes, what might be appropriate criteria for selecting as a positive control asbestos fibers with fiber size distribution similar to the test material?

Question 3: How many exposure concentrations of a positive control should be conducted? If only one exposure concentration is used, should it be comparable to the highest exposure concentration of the test material in terms of fiber concentration or fiber lung burden? Or should it be at an exposure concentration expected to induce tumor effects?

Conclusions and recommendations. A positive control need not be included in every study, but each new test system (including use of a different animal species or strain) should be validated with a positive control material.

In addition, it was generally agreed that a chronic multidose asbestos inhalation study in rats is critically needed to validate and calibrate the chronic rat inhalation assay (a) for evaluation of the toxic and carcinogenic potential and potency of other fibers and (b) for comparison with known human carcinogenicity data for asbestos. It was strongly recommended by the panelists that priority should be given to conduct such a multidose asbestos inhalation study. The exposure concentrations should be based on the outcome of a subchronic 90-day inhalation study using the same criteria for deriving the MAC and MTD as is used for the testing of other fibers.

II.6. Criteria for a Negative Inhalation Test

EPA generally considers an oncogenicity study to be negative if there is an absence of tumor effects in an adequately sensitive and well-conducted study. The key issue which needs to be defined is what constitutes "an adequately sensitive study" for the testing of fibrous particles.

Question: What might be suitable criteria for the acceptance of an inhalation study with fibers as negative (no tumors, achievement of a MTD, appropriately spaced lower concentrations, adequate animal survival, use of an appropriate positive control)?

Conclusions and recommendations. For acceptance of the results of a chronic inhalation exposure study with fibers as negative, the study must have been designed and conducted according to the criteria outlined previously; the health effects of concern must not be significantly more frequent in the exposure groups than in the control group. In order to detect a positive effect, the power of the study should be such that α as the type I error is controlled at 0.05 and as the type II error is controlled at 0.2.

III. Prechronic Studies

EPA generally recommends a subchronic 90-day study to help establish suitable study conditions for the chronic study, especially for setting appropriate exposure concentrations. The subchronic study would also provide important toxicologic information to be used in conjunction with results of other mechanistic studies to help in the interpretation of the chronic study findings.

It has been suggested that for the testing of particle toxicity, the primary goals of a subchronic study should include: (a) an evaluation of patterns of particle deposition (including hot spots of deposition), translocation, and clearance, and determination of the lung burden at which impaired clearance occurs; and (b) an evaluation of toxicity and mechanisms of pulmonary toxicity.

Question 1: Should a subchronic 90-day study be recommended prior to conducting the chronic study? Or should it be made optional?

Question 2: What are the primary goals of the subchronic study?

Question 3: What specific data related to fiber disposition should be obtained in the subchronic study?

Question 4: Are there any specific methods that should be recommended to measure the effects of fibers on lung clearance (e.g., use of radiolabeled particles)?

Conclusions and recommendations. A subchronic study should be conducted unless sufficient data are available from other studies to allow for the proper design of the chronic study. The primary goals of the subchronic study are (1) to establish lung burdens and potential target sites to aid in design of the chronic study and (2) to evaluate toxicity for a variety of important biological endpoints.

Lung burden analysis and bronchoalveolar lavage fluid (BALF) are recommended in prechronic inhalation studies of fibers (as well as in chronic studies, see sections below). Early fibrosis should be assessed through histological examination. Other studies would be complementary, such as replica cast studies to identify hot-spot locations of deposition; however, these studies are ancillary and should not be required.

Impairment of clearance should be assessed via challenge with a tagged particle. Clearance should be assessed after the 90-day exposure period. The clearance of the labeled particles should be measured over a period of a few months. Although no specific method is recommended, the method chosen should be validated. Also, it is important to distinguish between fiber clearance and clearance of the test particle used in the challenge.

IV. Fiber Disposition and Dosimetry

IV.1. Lung Burden Analysis

Lung burden analysis is not a requirement in EPA's current study protocol for chronic toxicity and oncogenicity testing of chemicals. However, lung burden data would provide useful data on biopersistence of the test fibers and serve as a better measure of internal dose.

Question 1: Should lung burden analysis be included in the subchronic and chronic studies?

Question 2: If yes, should the procedure as recommended by the International Cooperative Research Programme (ICRP) be adopted? There are data to indicate that fiber burden data based only on the accessory lung lobe (as recommended by the ICRP) may not be representative of the whole lung because of nonuniform pattern of deposition. In view of these findings, what changes should be recommended?

Question 3: Should any specific methods for lung ashing be recommended?

Question 4: How often should lung burden analysis be performed (at interim and final sacrifice time points)?

Conclusions and recommendations. Lung burden analysis should be included in the subchronic and chronic studies even if extra animals need to be added to the study. Data should be obtained on lung burdens (a) to assist in establishing the chronic exposure levels or aerosol generation changes needed to get more fibers deep into the lung and (b) to quantify aspects of risk assessment related to dosimetric adjustments before extrapolation. Data also should be obtained on fiber deposition in the nasal cavity and the fiber burden in the thoracic lymph nodes, and collection of pleural tissues is encouraged.

For fiber burden analysis, one of the two lungs (left or right) should be used, rather than only the accessory lobe (as recommended by the ICRP). It may be possible to determine a correction factor during subchronic studies that would allow the use of only one lobe in the chronic studies. However, disease development could change deposition patterns and invalidate a correction factor. Five to six animals per exposure group should be studied at each time point.

Rather than "lung ashing," the proper term is "lung digestion," because the guidelines will apply to other types of fibers in addition to man-made vitreous fibers. No specific lung digestion method is recommended. The investigator must show that the fibers are not affected by the method used to harvest them from the lung tissue.

Lung burden analyses should be required after 3, 6, 12, 18, and 24 months of exposure. Pleural burden analysis is not recommended at this time. However, in view of the potential use of the pleural data in quantitative risk assessment and the cost of repeating studies, investigators should be encouraged to collect pleural burden samples and keep them available for future analysis.

IV.2. Biomarkers of Toxicologic Effects

Recent studies in rats have demonstrated the value of BALF analysis in subchronic studies in predicting the chronic effects of exposure to a variety of particles. This will enable the investigator to better select the exposure concentrations for the chronic studies as well as to help in understanding the biochemical and cellular sequence of events of particle-induced toxicity and carcinogenicity.

Question 1: Which specific biomarkers of toxicity and carcinogenicity should be measured in the subchronic study (e.g., BALF analysis, cytotoxicity, cell proliferation)?

Question 2: Should BALF analysis be made mandatory for the chronic study?

Question 3: If yes, should the procedure as specified in the ICRP's protocol be adopted? Are there any modifications that should be considered?

Conclusions and recommendations. The subchronic study should include analysis of BALF for evaluation of inflammatory response (e.g., protein content, enzymes, presence of inflammatory cells) and measurement of cell proliferation. BALF analysis should be required in the chronic study.

IV.3. Dosimetry and Interspecies Considerations

The size distribution of fibers that deposit in the lungs of rodents may be different from those in the lungs of humans because of anatomical and physiological differences (e.g., lung surface area, respiratory exchange rates, clearance rates, dissolution rates). Deposition and clearance mathematical models have been developed to relate the fiber lung burden to biological effects which may serve as useful exposure-dose–response models for human risk assessment.

Question 1: Which additional dosimetry information could be obtained as part of the chronic study to help in characterizing the human hazard and exposure-dose–response assessment?

Question 2: With regard to the selection of the human dosimeter, is milligrams per kilogram body weight adjusted for differences in metabolic rate appropriate for fibers? For diesel exhaust, EPA utilizes milligrams per unit alveolar surface area as the dosimeter without an adjustment for metabolic rate. Is this reasonable for fibers, or are their mechanisms sufficiently different?

Conclusions and recommendations. Quantitative information on the fiber burden at airway bifurcations or other localized sites would be useful. To the fullest extent possible, tissues should be preserved in such a way that other measurements or analyses can be conducted later, depending on the outcome of the bioassay or development of new molecular or biochemical techniques.

The expert panel did not endorse use of milligrams per kilogram body weight as a viable human dosimeter, even after adjustment for species differences in metabolic rates. Various dose metrics can be computed, but none of them affect the design of subchronic or chronic studies.

V. Mechanistic Studies

The mechanisms by which fibers induce fibrosis and cancer are not known, but are thought to be mediated via reactive oxygen species and growth factor pathways. Fibrous particles may also cause mutagenic events through induction of DNA strand breaks, clastogenic effects, deletions, and interference with the spindle apparatus of dividing cells. Mechanistic studies are generally not required as part of EPA's test guidelines. However, in assessing the potential chronic toxicity and carcinogenic effects of respirable fibers in humans, it is desirable to consider the differences of species responses and the understanding of the mechanisms of fiber-induced toxicity and carcinogenicity. This may allow an improved basis for extrapolating observed effects in the test species exposed to high concentrations to humans exposed to relatively lower concentrations generally found in the workplace and the general environment.

In vivo and *in vitro* mechanistic studies have been developed and proven useful and valuable in elucidating the mechanisms of fiber-induced pathogenesis, i.e., chronic pulmonary inflammation, fibrogenesis, and oncogenesis. Evaluative endpoints may include cytotoxicity, phagocytosis, cell proliferation, expression and induction of specific mediators (e.g., cytokines, growth factors, antioxidants), unscheduled DNA synthesis, determination of DNA repair, or mutational frequencies of target cells (e.g., HRPT mutation assays of type II cells).

Question: Should any mechanistic studies be recommended? Which and when (prior to the chronic study, in parallel with the chronic study, and/or subsequent to the chronic study)?

Conclusions and recommendations. No mechanistic studies are recommended at this time. However, investigators should be encouraged to obtain mechanistic information as far as possible during the course of subchronic or chronic inhalation studies. A high research priority should be to determine whether fiber carcinogenesis is a direct effect or an indirect effect related to

inflammation. It was suggested that the most promising approach for obtaining mechanistic information is to isolate target cells after *in vivo* exposure for use in subsequent *in vitro* studies. Other priorities for research include (1) development of short-term *in vivo* assays with *ex vivo*/*in vitro* investigations in appropriate target cell populations, (2) investigation of oncogenes and tumor suppressor genes in human and rodent tumors, (3) development of transgenic animal models, (4) species comparisons of fiber-induced pulmonary effects *in vivo* and *in vitro,* and (5) use of pleural lavage to evaluate predictive markers of response.

VI. Histopathologic Evaluation

In this session, in addition to the following questions raised in the issue paper, the panel discussed what needs to be examined and measured in the histopathological evaluation.

The use of the Wagner scoring system has been suggested at the Chemical Industry Institute of Toxicology (CIIT) workshop (McClellan *et al.,* 1992) for the evaluation of pulmonary fibrosis to enable direct comparison of effects induced by different types of fibers. The Wagner scoring system has also been utilized in recent studies on synthetic mineral fibers. This system, however, does not consider the mass of the lung tissue involved. One method that has been used by other investigators includes a morphometric approach to determine the percentage of lung tissue involved in fibrotic lesions. In another method, total lung collagen is measured as an indicator of lung fibrosis.

Question 1: Is it necessary to utilize a standardized scoring system for the evaluation of cellular changes and fibrosis in the lung?

Question 2: The International Cooperative Research Programme (cited in USEPA, 1996) has recommended that the Wagner scoring system be revised to take into account the limitation [that it does not consider the mass of the lung tissue involved]. What specific modifications must be made before it can be adopted for inclusion in the test guidelines?

Conclusions and recommendations. In the chronic study, use of the Wagner scoring system to evaluate progression of fibrosis has the disadvantage of being purely qualitative and inconsistently applied. To promote more quantitative evaluation, the testing guidelines should specify set procedures for grading of lesions and for lung preparation. Further research is needed before other quantitative histopathological methods can be recommended for large-scale testing. However, a promising approach could be quantitation of collagen deposits using sirius red and evaluation with polarized light. Neoplastic endpoints recorded should include epithelial hyperplasia, alveolar bronchiolization, metaplasia, adenomas, mesotheliomas, and carcinomas. Keratin cysts should be identified as such, to permit subsequent evaluation, and it must be stated whether or not a cyst presents evidence of invasion or dysplasia. A dissecting microscope should be used to examine for mesotheliomas. In distinguishing between hyperplasia and mesothelioma, standard diagnostic criteria should be applied to identified lesions. Established published guidelines on the use of blinding in histopathology should be followed, e.g., those published by the Society of American Pathologists.

VII. Screening Battery

Numerous test systems and/or protocols have been developed and utilized by the scientific community for evaluating the fibrogenic and carcinogenic potential of fibrous particles. However, there has been considerable debate about the scientific validity and utility of available test methods. This subject along with research needs for better understanding of the mechanisms of fiber-induced disease have been the topics of discussion at several scientific conferences, workshops, and expert meetings, sponsored by various organizations (e.g., Dement, 1990; WHO, 1992; McClellan *et al.,* 1992; ISRTP, 1994). A tiered approach for evaluating the toxicity and carcinogenicity of new fibers or untested fibers has also been recommended at a workshop sponsored by the CIIT as a guideline for research purposes (McClellan *et al.,* 1992).

Other organizations such as the ICRP have recently developed a draft protocol for the assessment of synthetic fiber's toxicity, as a part of a tiered-approach testing program (see USEPA, 1996).

Question 1: Recognizing that no single screening study can accurately predict the *in vivo* responses from long-term exposure to fibers, after evaluating the physical and chemical properties of the fibers (Tier I), can Tier II and Tier III types of studies as defined in the CITT workshop proceedings (McClellan *et al.,* 1992) be used to screen and set priorities with regard to confirmatory testing in a chronic study to obtain more definitive information for risk assessment purposes? If not, why not?

Question 2: If yes, what specific tests or combinations of tests can be utilized in this screening battery?

Question 3: Given that *in vivo* studies using noninhalation methods of exposure (e.g., intraperitoneal injection, intratracheal instillation studies) have been proven useful in identifying the potential health hazard to humans, should they be considered acceptable as an alternative screening test or an adjunct to short-term inhalation studies in a screening battery?

Question 4: Intracavitary testing (ip study) of fibers has been proposed by the ICRP (Oberdorster, 1995; see USEPA, 1996). Would a positive finding using this protocol constitute a potential hazard to humans, or would a positive finding need to be followed by a chronic inhalation study to confirm the hazard?

Conclusions and recommendations. Appropriately designed Tier II (*in vitro*) and Tier III (short-term *in vivo*) studies can provide useful information to assess the relative potential of fibrous materials to cause toxicity in the lung and associated tissues. Along with other information, data from a battery of Tier II and III studies can provide key information to prioritize materials for further chronic testing. At present, no single assay or battery of short-term assays can predict the outcome of a chronic inhalation bioassay with respect to carcinogenic effects.

Tier II in vitro tests. Solubility/durability can influence the lung's response to long-term inhalation of fibers. In most instances, rates of *in vitro* and *in vivo* solubility correlate well, although the absolute rates may differ. Therefore, *in vitro* assays providing information on fiber solubility/durability can provide useful information for prioritizing groups of fibers for further testing. It was suggested that *in vitro* solubility alone could not be used to rule out further testing, and that fibers should be evaluated in an *in vivo* test system for toxicity and *in vivo* dissolution. Although other characteristics of fibers that can be examined in acellular *in vitro* tests were discussed, no general agreement was reached on their value as routine tests for assessing the potential of fibers to cause toxicity. *In vitro* cell or tissue culture assays can potentially provide useful information on fiber toxicity; however, these systems are not yet well enough validated or understood to be recommended for routine use in screening fibers toxicity.

Tier III (short-term in vivo studies). There is no standardized protocol for short-term respiratory tract exposures to fibers (i.e., less than 3-month exposure) followed by characterization of the lung response periodically over several weeks. Nonetheless, this type of study is a useful tool for assessing the relative ability of fibers to produce nonneoplastic effects (e.g., inflammation, cell proliferation, fibrosis) in the lung. Thus, information from short-term *in vivo* studies, combined with data from Tier I studies (physicochemical properties), would be useful for prioritizing materials for longer-term studies. Short-term screening studies should include (but not be limited to) analysis of BALF for markers of cell injury and inflammation, histopathology, and assessment of lung fiber dose. Study design should include assessments of dose–response relationships and, when possible, comparisons to physically and chemically similar "control" fibers for which chronic lung effects already have been evaluated. Exposure concentrations should include at least one level that elicits significant lung effects, to provide a basis for comparison of both the nature and persistence of the response to the fibers. In addition, it would be useful to assess fiber biopersistence following a short-term exposure (e.g., a 5-day exposure, followed by monitoring for several weeks).

Intratracheal instillation. Intratracheal instillation was considered by a majority of the panel members to be an acceptable alternative to inhalation exposure for short-term screening studies to assess the relative biopersistence and relative nonneoplastic toxicity of fibers in the lung provided that low doses are used. Intratracheal instillation allows a known amount of test material to be administered to the lung in a manner not requiring the resources (i.e., exposure facility and level of research funding) needed for inhalation exposure. Moreover, human-respirable fibers not respirable by the rat may be evaluated via intratracheal instillation, although care should be taken to avoid higher doses of longer fibers which may result in clumping. However, the intratracheal instillation delivers materials at a much higher dose rate than does inhalation, and care must be taken to ensure that the responses observed after intratracheal instillation are comparable to what would be expected after inhalation. Fiber doses should be low enough to minimize problems of fiber clumping and overwhelming of lung defense mechanisms. It would be useful to include a "control" fiber for which the lung tissue response after inhalation has already been characterized to demonstrate that intratracheal instillation produces a response similar to that expected after inhalation. The majority thought that intratracheal instillation should not be recommended for assessing the carcinogenic potency in long-term studies.

Intraperitoneal injection. Intraperitoneal injection studies can provide information on the interaction of fibers with mesothelial cells. However, for screening or rank ordering the potential toxicity of fibers in the lung based on intraperitoneal injection studies, the behavior in the lung (e.g., clearance, translocation) of the fibers being evaluated must be taken into account. What constitutes a MTD for ip study remains to be defined. There was little discussion on this subject.

CONCLUSIONS

EPA's health effects test guidelines for oncogenicity and combined chronic toxicity and oncogenicity are widely accepted by the scientific and regulatory communities for the testing of chemical substances (EPA, 1992). EPA's guidelines are similar to those of the Organization for Economics Cooperation and Development (OECD) and the National Toxicology Program (NTP). It is recognized, however, that these guidelines need to be modified to take into account testing issues which are unique to fibrous particles.

Numerous test systems and/or protocols (e.g., McClellan, 1992, WHO, 1992) have been developed and utilized by the scientific community for evaluating the

fibrogenic and carcinogenic potential of fibrous particles. However, there has been considerable debate about the scientific validity and utility of available test methods. There are also some differences between these study protocols and the EPA's test protocols with regard to certain standard requirements of the design of the study (e.g., species, strain, gender, exposure method). Thus, there is a need for examining and articulating the scientific bases for any recommended changes for the testing of fibrous particles.

EPA, in collaboration with the National Institute of Environmental Health Sciences (NIEHS), the National Institute for Occupational Safety and Health (NIOSH), and the Occupational Safety and Health Administration (OSHA) through an interagency working group, has identified a number of scientific issues related to fiber testing that requires further evaluation by expert scientists.

In this workshop, the expert panel concluded that at present, no single assay or battery of short-term assays can predict the outcome of a chronic inhalation bioassay with respect to carcinogenic effects; a number of guidances specific for the design and conduct of prechronic and chronic inhalation studies of fibers in laboratory rodents were recommended. Overall, the objective of the workshop has been accomplished. The panel recommendations shall be considered for incorporation into a proposed health effects test guidelines being developed by EPA.

APPENDIX

Expert Panel

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