

TOXICOLOGICAL STUDIES IN A MULTI-SITE AMBIENT PM RESEARCH EFFORT: BACKGROUND AND A BASIS FOR DISCUSSION

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Introduction: Putting Toxicology Studies in Perspective

In their final report, the NRC Committee on Research Priorities for Airborne Particulate Matter (NRC, 2004) indicates that in order to address the toxicity of different components of the particulate matter mix in ambient air, that "...coherent and converging evidence is needed from both toxicological and epidemiological research that addresses specific components and health outcomes in parallel."

The role of toxicology in any PM research effort is the same as that for epidemiology, namely to provide relevant health effects data as input to the standard's setting process. To this end, toxicology can establish cause-effect relationships for exposures to specific materials or mixtures of materials at specific levels of exposure, i.e., biological plausibility, and can develop exposure-dose-response profiles. Such studies are needed since epidemiological studies can show statistical associations between health outcomes and some metric of exposure, but they cannot establish a definitive cause-effect relationship between exposure and response.

Toxicological studies have both advantages and disadvantages. Controlled exposure studies provide the ability to directly evaluate the influence of various host factors, such as age, gender, or pre-existing disease on response without the complication of various confounding variables. While studies of this type can be done with humans, there are strict limitations in study design, such as exposure duration and types of subjects used. Thus, a common problem in toxicology is the need for interspecies extrapolation when studies are performed in nonhuman animal species.

What epidemiological studies can do is clearly evaluate responses to realistic exposure scenarios in real world populations. Such conditions can only be simulated, sometimes quite poorly, by toxicological evaluations. However, the former cannot always provide accurate or complete assessment of actual exposures, often relying on a limited number of measurements made at sites that are often removed from the exposure groups of concern. Furthermore, epidemiological studies cannot always separate biological responses due to specific pollutants because of co-correlation, and various other confounding factors cannot always be adequately controlled for in such studies. Finally, they can study only a limited number of responses, which may not include the most sensitive marker of potential disease or dysfunction. On the other hand, toxicological studies can be used to assess the relative contribution to measured response(s) of components of pollutant mixtures, and to evaluate the role of potential confounders in

response. Furthermore, toxicological studies allow for precise control over exposure concentrations and durations.

Given the above considerations, what would be the role of toxicology in a multi-site PM research effort? When epidemiological evidence is ambiguous, toxicology can provide needed mechanistic support to strengthen the scientific arguments for a specific ambient concentration and/or exposure duration as being critical for initiation of a response of interest. On the other hand, when epidemiological evidence is overwhelming and strong enough to provide substantial support for some standard, toxicology can provide validation of coherent mechanisms underlying the evidence upon which the epidemiological conclusions are based, thus providing a margin of comfort for the conclusions reached.

Some General Considerations in Designing a Multi-Site Exposure Study

A number of scientific issues need to be addressed when incorporating toxicological studies into a multi-site research effort. These issues are related to the type of exposure being proposed, namely *in vivo* and *in vitro*.

In Vivo Exposures

One of the issues in evaluating susceptibility of different human or animal populations to PM involves the question of whether increased susceptibility is due to differences in dosimetry of inhaled PM, to innate differences in susceptibility, or perhaps to both. Compounding this is the ever present issue of dosimetric extrapolation between humans and animals used in toxicological studies. This is important, since clearance and translocation mechanisms, as well as potential sites of PM activity, are likely related to sites of deposition, which can differ substantially between different species, as well as between normal and “compromised” individuals within one species. Thus, *in vivo* exposure studies must incorporate some measure of dosimetry if there is going to be an attempt to compare results from different laboratories and using different types of particles or ambient PM with different size characteristics.

An issue related to dosimetry is the most relevant, to health outcomes, exposure metric to use in evaluating exposure-response relationships derived from *in vivo* exposure studies. There is some evidence, at least for certain particle size fractions, that number concentration may be an important metric in this regard. Thus, both mass and number concentration should be evaluated, especially in studies in which ultrafine particles are included in the exposure atmosphere. Very often, studies are performed by different investigators using similar mass concentrations of particles in different size ranges, and in such cases there can be large differences in the number concentration, making it difficult to compare responses.

Controlled exposures to humans and animals *in vivo* may involve production of exposure atmospheres by various techniques. These have generally included resuspension of ambient PM collected on filters, laboratory generation of surrogate

particles representing specific components of ambient PM, and actual ambient fine fraction PM that has been concentrated (CAPs). In terms of application to a multi-city study, the latter procedure is recommended.

The use of CAPs has the advantage of exposing either humans or animals to “real world” ambient PM. A disadvantage is that the physicochemical nature of the exposure atmosphere has temporal variability. However, this would be the same variability to which the general population is exposed. As long as there is detailed characterization of the atmospheres during each exposure period, this temporal variation can be used in attempts to tease out the more or less toxic components of the ambient PM mix. Based upon these results, controlled exposures with certain surrogate PM can then be performed in a further attempt to refine understanding of specific components related to toxicity of ambient PM. Another shortcoming of CAPs is that the range of concentrated particles in the exposure atmosphere is somewhat limited; for example, the devices generally will concentrate particles in the 0.5 – 2.5 μm range with smaller ones coming through at ambient levels and larger ones generally removed prior to exposure.

When using particle concentrators for exposure, the decision needs to be made as to whether to uniformly scrub out or leave in ambient gases, such as sulfur dioxide, nitrogen dioxide and, perhaps most importantly in terms of potential interaction, ozone. Whatever is decided, the methodology needs to be consistent across all sites. Given the importance of PM-gas pollutant interactions, it is recommended that at least some of the exposure studies include ambient gases, especially ozone.

There are various biological endpoints that could be examined in these studies, but all should be relevant to understanding health outcomes noted in epidemiological studies and in devising hypotheses for biological plausibility for these outcomes. Some of the endpoints should be comparable to those measured *in vitro*, such as reactive oxygen species and cytokines in lavage fluid. Others should relate to health outcomes noted in epidemiological studies, such as changes in cardiac physiology and alterations in immune function in the respiratory tract.

In terms of exposure duration, human studies are generally very limited in this regard. Some animal studies should mimic these human exposures, while others should be conducted for several hours per day, and for several days per week. A combination of acute and chronic durations may allow for understanding differences in short vs. long term exposures to ambient PM noted in epidemiological studies.

The animal models and human subjects used should include those that are deemed to represent compromised human populations. All such models should be clearly characterized in terms of their relevance to human disease.

Table 1 summarizes the various exposure populations and biological endpoints used in inhalation studies with CAPs as reported in the literature.

In Vitro Exposures

Studies using *in vitro* experimental systems are generally performed to help in determining pathophysiological mechanisms of response and, as such, are a useful complement to *in vivo* controlled exposure studies. On the other hand, the utility of such studies is limited by the difficulty in extrapolating the results to the “whole animal.” For example, while *in vitro* studies can be used to assess effects of PM constituents on one cell or tissue type, these latter do not exist in isolation *in vivo*, and one type of tissue will often modify effects in another following *in vivo* exposure. Furthermore, the relationship between delivered dose *in vitro* to delivered dose *in vivo* is often ignored, or cannot be determined. Furthermore, the method of PM delivery is not always relevant to the “real world.” For example, delivery via a system that exposes the model to airborne particles may be more realistic than exposure to some suspension in tissue media. All this being said, *in vitro* studies are useful as a component of a larger study designed to examine a specific hypothesis of effect; they should generally not be used as the sole basis for development of any mechanistic hypothesis for PM action. In any case, thought has to also be given to the likelihood that collection and regeneration of any particulate matter will result in physicochemical alterations to the material, which may result in it being quite dissimilar to the material as it would be inhaled from ambient air by a population of concern.

Given these caveats, air samples from a multi-city study could provide valuable material that could be used in assays that would provide an indication of “relative” toxicity of materials from different sites. This type of study would involve exposure of cells or cell lines to PM samples obtained from the different sites, and using a similar exposure concentration paradigm and examining the same endpoints. The endpoints selected should be those that would help in determining mechanisms of responses observed with *in vivo* exposures, or from epidemiological studies, and could indicate some basis for different qualitative responses from ambient exposures at different sites based upon effects of specific cell/tissue lines

Various approaches can be used in terms of the specific exposure material. In order to attempt to speciate material so as to provide a better understanding of which components may be more or less toxic, *in vitro* exposures could be to aqueous extracts of the ambient PM in the same size fraction obtained from the different sites, which would include the soluble metals, or to water insoluble components or to whole PM in suspension. Differences in responses to each of these may help in elucidating toxicity of specific constituents of PM from different areas. In addition, *in vitro* exposures to different size fractions, such as fine and coarse, from the different areas, if available, could also be done in this regard. This would provide a matrix of size/chemical composition from the different sites on the same endpoints at the same “exposure” concentration. Whatever the experimental design, it would be critical that the PM samples be handled similarly in each of the laboratories performing the exposures and that exposures be performed in a comparable system. Furthermore, exposure – response paradigms should be provided in these studies.

Table 2 summarizes the various exposure models and biological endpoints used in exposure studies with ambient PM extracts, which would be the *in vitro* exposure “equivalent” of CAPs or components of CAPs, as reported in the literature.

Development of an Exposure-Response Matrix

The ultimate goal of the toxicological effort in conjunction with a multi-site study is to enhance our understanding of the physicochemical and/or source characteristics of ambient PM that may contribute to adverse health outcomes. The goal is to develop a matrix of exposure-response profiles based upon a multidisciplinary approach of *in vivo* and *in vitro* exposures, taking advantage of the strengths of each, as previously noted. By attempting to standardize exposure design and endpoints, one should be able to develop a matrix of PM component-response characteristics, looking at commonalities between effects noted in each type of study and implicating specific PM characteristics in adverse health effects. This could then lead to further study of specific components using refined surrogate atmospheres and specific animal or *in vitro* models aimed at addressing more refined questions that would strengthen the hypothesized mechanistic pathway of PM effects, both systemically and on the respiratory tract.

A “Model” Multi-Site Toxicology Study

Given the issues discussed about for consideration in designing any study, the following represents a broad outline of a toxicology study using ambient PM obtained from various sites. The basic study parameters are provided in Table 3. This represents an “ideal” study, i.e., what would be most useful to include to provide information related to the role of specific chemical species in health effects outcomes noted with exposure to ambient PM.

The study would involve concentrators at each of the sites so as to provide “real world” real-time ambient particulates for *in vivo* inhalation exposures and samples for *in vitro* exposures. The exposures should be to concentrated fine particulates, including the ambient gases normally present at each site.

The “ideal” study would involve both animal models and human subjects. The animal studies would be for longer duration than the human studies, but the latter would be used to help evaluate the correspondence of effects in animals and humans with a short-term exposure. Thus, for example, biological assays performed with the animals after the first day of exposure would be compared to those obtained in the human subjects exposed for a single 3 hr time period.

The animals used involve both normal adults as “controls”, as well as two models of susceptible human groups. The latter could consist of old animals, to assess age-related differences in response, and a model of a relevant human disease state, such as the SH (spontaneously hypertensive) rat, a model for cardiac hypertrophy (which increases risk for further cardiac disease). The human subjects would be normal young adults,

asthmatics as a model of pulmonary disease and a group with mild cardiovascular disease. The biological assays selected for the *in vivo* exposure will be based upon direct or indirect correspondence to health outcomes noted in epidemiological studies involving ambient PM exposures.

As noted, *in vitro* exposures are a useful adjunct to the *in vivo* exposures. Some of the "exposure atmospheres" used could be water soluble extracts and total organic extracts of ambient PM collected on filters and the exposure models could be bronchial epithelial cell lines and macrophage cell lines or macrophages recovered by lavage, to assess effects on specific cells that may help explain some of the effects noted in the *in vivo* exposures. Macrophages and perhaps other cell types could be obtained from the compromised animal models. The exposure system should consist of cells bathed in media on their basal side and exposed to the specific PM extract on their apical side. If resuspended ambient PM is used, the exposure device should consist of a unit that aerosolizes the particles for delivery to the cells.

TABLE 1

**SUMMARY OF INHALATION EXPOSURE MODELS AND BIOLOGICAL
ENDPOINTS USING CAPs**

Human Exposures

Subjects

Normal adults (18-40 yr)
Elderly adults (>60 yr)
Asthmatic adults

Range of Exposure Durations

2 –3 hr, some with exercise

Biological Endpoints

Pulmonary

Lavage parameters – cell differentials, cell viability, etc
Pulmonary function
Cell adhesion molecule levels
Inflammatory markers – IL-6, IL-8, IL-10

Cardiovascular

ECG analysis (Holter)
Peripheral blood cytokines (IL-6, IL-8)
Peripheral blood fibrinogen level
Brachial arterial diameter
Peripheral blood cell differential

Animal Exposures

Exposure Model

Normal rats
Bronchitic rats (SO₂ induced)
Pulmonary hypertensive rats (monocrotaline induced)
Cardiomyopathic hamsters
Dogs (normal and with occluded coronary artery)

Range of Exposure Durations

Single 1 hr exposure up to 6 hr/d exposures for 3 d

Biological Endpoints

Pulmonary

Lavage parameters – cell differentials, cell viability, TNF α , cytokines etc.

Pulmonary function

RNA analysis in lung tissue and lavage for mRNA

Reactive oxygen species production by macrophages

Pulmonary Immunological

Bacterial burdens of instilled microbes

Bacterial killing

Cardiovascular

Pulmonary artery lumen size (vasoconstriction assay)

Peripheral blood differentials

EKG analysis

TABLE 2

SUMMARY OF *IN VITRO* EXPOSURE MODELS AND BIOLOGICAL ENDPOINTS USING AMBIENT AIR-DERIVED PM

Particles Used

Aqueous extracts of ambient PM

Total or various residual organic solvent extracts of ambient PM

Exposure Models

Human epithelial cell line (BEAS-2, A549)

Human neutrophils from lavage

Human alveolar macrophages from lavage

Murine alveolar macrophages from lavage

Murine macrophage cell line (RAW264.7)

Myofibroblasts

Biological Endpoints

Cytokine release

Activation of NF- κ B

Macrophage phagocytic activity

Cell signaling activity

Reactive oxygen species release

Heme-oxygenase expression (oxidative stress marker)

Intracellular glutathione levels (oxidative stress marker)

TABLE 3

**OUTLINE OF DESIGN FOR “IDEAL” MULTI-SITE TOXICOLOGICAL
INHALATION STUDY**

Ambient Particle Collection/Exposure System

Fine particle concentrator (0.1 – 2.5 μm) at each site

Ambient gases will not be scrubbed from the exposure system

Particle Exposure Metric

Mass and number concentration on a size fraction basis

Chemical Analysis of Exposure Atmosphere

Particles

Elemental carbon

Organic carbon

Sulfate

Nitrate

Acidity

Trace metals (transition metals)

Gases

CO

NO₂

SO₂

O₃

Exposure Design

Animal Studies

Three exposure groups for each exposure model:

-Control (Clean air filtered for particles and scrubbed for gases)

-Ambient Gases Only (Ambient air with PM filtered out)

-Total Ambient Air (Exposure to air from CAPs device with PM and gases intact)

Exposures for 3 hr/d for up to 30 consecutive days in each season (winter, summer) at each site. Subgroups of animals sacrificed immediately after 1, 15 and 30 days of exposure and then at 15 days after the last exposure to assess any “long-lasting” effects.

Total of 40 animals in each exposure group, with 10 sacrificed at each noted interval.

Human Studies

Three exposure groups for each exposure model as above for animal exposures.

Each exposure group consists of 10 subjects.

Single, 3 hr exposures performed twice in each season at each site for each subject.

Exposure Models

Animal Studies

Normal rats (young adult)

Compromised models

Old rats (e.g., NIH aged rats) – model for aged human population

SH (spontaneous hypertensive) rats – model of cardiac hypertrophy

Human Studies

Normal, healthy subjects (adults aged 18 – 50)

Compromised subjects

Asthmatics (pulmonary compromise)

History of mild cardiac disease (cardiovascular compromise)

Biological Endpoints

(NOTE: H indicates assay for human studies; A indicates assay for animal studies)

Pulmonary

Physiological

Pulmonary function (H, A)

Biochemical/Cellular – markers of inflammation/injury

Lavage analysis (A)

Cell differentials

Cell viability

Total protein

LDH

Reactive oxygen species from macrophages

Cytokines

Histology (A)

General pathologic assessment of airways and parenchyma

Cardiovascular

Cardiac Physiology (H, A)

EKG analysis (animal via telemetry, human via Holter)

HRV

Interval analysis

Beat rate

Waveform analysis

Cardiac Histology (A)

Assessment of lesions related to inflammation, necrosis, etc.

Systemic Blood (H, A)

Plasma

C reactive protein (nonspecific inflammatory marker)

Fibrinogen and/or clotting factors

Formed elements

Cell differentials

Platelet adherence/activation

Reactive oxygen species for leukocytes

Dosimetry

In animal studies, a few animals in each group will be used to examine exhaled vs inhaled mass/number concentration of CAPs.