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Establishing Aerosol Exposure Concentrations for Inhalation Toxicity Studies

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Establishing Aerosol Exposure Concentrations for Inhalation Toxicity Studies. LEWIS, T. R., MORROW, P. E., MCCLELLAN, R. O., RAABE, O. G., KENNEDY, G. L., SCHWETZ, B. A., GOEHL, T. J., ROYCROFT, J. H. (1989), AND CHHABRA, R. S. *Toxicol. Appl. Pharmacol.* **99**, 377–383. Criteria for the selection of aerosol concentrations to be used in inhalation studies assessing the toxicity and carcinogenicity of chemical substances were discussed by the authors in a meeting sponsored by the National Toxicology Program. Concepts in the design of aerosol inhalation studies emerged from that meeting and are being communicated through this publication. Inhalation studies assessing the toxicity and carcinogenicity of aerosols have often used maximum exposure levels on the basis of technological feasibility. Evidence has now accumulated that the amount of pulmonary burden of deposited particles impacts on particle clearance above some as yet not well-defined exposure concentration. The sequelae are such that lung clearance decreases with increased particulate burden to the point of approaching complete cessation. This paper focuses on the major determinants in establishing maximal aerosol concentrations for use in inhalation toxicity studies with special emphasis on experimental design features to assess lung retention. The subject matter of this paper is a rapidly developing area in terms of knowledge. Accordingly, the contents of this article are intended as guidelines and not as absolute rules for the conduct and interpretation of inhalation exposure studies.

Previous guidelines or recommendations for the conduct of inhalation toxicology and carcinogenesis studies have not fully addressed the subject of the maximal aerosol exposure concentrations to be employed (EPA, 1982ab; OECD, 1981; NTP, 1987). As part of its mandate to address issues related to the design of toxicologic studies, the Toxicology Design Review Committee (TRDC) of the National Toxicology Program (NTP) convened a meeting to address this particular topic. The nine participants (four non-NTP national experts in inhalation toxicology representing academe, national laboratories and industry, and five NTP scientists) focused their attention on the major determinants to establishing maximum aerosol concentrations in inhalation toxicity studies. Special emphasis was placed on substances which are relatively insoluble and of low order of systemic and respiratory toxicity. It was not expected, however, that the discussants would resolve all the issues inherent to the design and conduct of prechronic and chronic aero-

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sol inhalation toxicity studies. The paramount considerations in the design, conduct, and interpretation of inhalation studies of aerosols discussed were the interrelationships among exposure concentrations, dose to the respiratory tract, and the subsequent toxicologic responses in the test subjects. Emphasis was placed on *in vivo* physiologically based pharmacokinetic factors as determinants of dose delivered to a specific tissue site and the resultant biologic responses. Issues related to the mechanics of aerosol generation and physicochemical modifiers of the aerosol concentrations within the exposure chambers such as, aerosol losses in the exposure system or to the animals' fur and contamination of the test atmosphere by products of animal excreta, were not addressed.

DOSE TO THE RESPIRATORY TRACT

The conduct of inhalation studies employing aerosols will involve substances that vary widely in their pulmonary retention. Those with high dissolution rates in tissue fluids (quite soluble) will usually be rapidly cleared from respiratory tract tissues and be translocated to other tissues or excreted. In this paper, pulmonary clearance is defined as a defensive function of the lung involved with the removal of foreign substances via structures as the mucociliary system, alevolar macrophages and the lymphatic system and is dependent upon such factors as absorption, solubility and particle size. Even with chronic exposure, respiratory tract tissues may never accumulate large concentrations. In such cases, respiratory tract toxicity may or may not be evident. Many particles that are quite soluble will also have appreciable absorption from the gastrointestinal tract for that portion of the inhaled material that is cleared from the respiratory tract to the oropharynx and swallowed. Establishment of exposure concentrations and the resultant doses to tissues in chronic aerosol inhalation studies is inherently less complex when systemic effects are dominant and can be related to differing dosage regimens.

Other substances may be quite insoluble in tissue fluids and are thus cleared relatively rapidly from the nasopharynx and tracheobronchial regions by mechanical processes and cleared slowly from the pulmonary compartment of the respiratory tract by several processes, including dissolution. For such compounds, the pulmonary burden of particles increases with exposure concentration and duration of exposure. Test protocols for aerosols that are to be employed in subchronic and chronic inhalation exposure, toxicologic, and carcinogenic studies should take into consideration the expected lung burdens with increased exposure concentration or duration. Accordingly, lung burdens of a test aerosol should be estimated, if not measured, in inhalation studies. This is especially important in chronic studies in which the test chemical is relatively nontoxic but high-level exposures may overwhelm pulmonary clearance mechanisms.

ASSESSMENT OF PULMONARY DUST RETENTION AND METABOLIC OVERLOAD

Interpretation of the results of inhalation toxicity studies, and especially of the presence or absence of pulmonary pathology, requires a knowledge of the lung burden of inhaled test material throughout the exposure and postexposure observation periods in the test species. For most inhalation studies, the test species are the rat and the mouse. The burden of relatively insoluble materials in the respiratory tract, and in particular in the lung, may be greater than predicted at higher levels of chronic exposure because of impairment of the pulmonary clearance processes.

The use of brief exposure to a radiolabeled particulate probe to monitor lung clearance rates can be useful in determining the lung burden of a test material which produces

overloading. Exposure to the aerosol probe can be used to help determine and interpret study conditions which avoid this problem. There is no one radiolabeled particulate tracer that may be applicable to all aerosol inhalation studies. It is particularly useful that the probe and test aerosol be of equivalent size. An excellent monitoring dust candidate is radiolabeled iron oxide due to its ready availability and low cost, the stability of the label and the ease of generation of specific particle sizes; radiolabeled polystyrene or fused aluminosilicate particles are alternate candidates. Such particles, when inhaled briefly, at low concentrations, have very reproducible pulmonary retention over a wide range of lung burdens and may be useful in assessing changes in the ability of the lung to clear particles. Serial measurements of the retention of the radiolabeled particles can be made rapidly with relatively inexpensive equipment. Such measurements may provide valuable insight into the presence or absence of impaired clearance. Appropriately sized iron oxide aerosols are easy to prepare and with ^{59}Fe providing a surrogate compound subject to noninvasive external measurement. These radiolabeled particles are largely (>90%) removed slowly from the lungs by macrophage-mediated clearance (Gibb and Morrow, 1962; Lehnert and Morrow, 1985). Suitable radiolabeled polystyrene particles are commercially available, e.g., 3M.

Several laboratories are currently measuring pulmonary dust retention following a brief exposure to a radiolabeled test aerosol at various times during prechronic or chronic studies of inhaled dust (Muhle *et al.*, 1988; Wolff *et al.*, 1987).

STUDY DESIGN FEATURES

A minimal of three concentrations of the airborne test aerosol should be used in chronic inhalation studies to establish dose–response relationship of the test substance. In general, the highest concentration of test material used should produce only minimal interference with the lung defense mechanisms as judged by impaired particulate clearance. Optimally, the lower exposure concentrations should not result in altered patterns of particle clearance and accumulation.

The fractional deposition of an aerosol in various regions of the respiratory tract of any species is dependent upon the aerosol size distribution and species differences in anatomical and physiological characteristics. Aerosols with a mass median aerodynamic diameter (MMAD) of less than 2 μm and a geometric standard deviation of less than 2.0 will have the highest fractional deposition in the lower respiratory tract of rodent species (Raabe *et al.*, 1977). Aerosols with a larger MMAD will have increasingly larger fractions of the material deposited in the upper respiratory tract and, correspondingly, less deposited in the lower respiratory tract.

Aerosol exposure concentrations in the chronic study can be selected on the basis of at least two considerations, i.e., modeling and experimental determination. For relatively insoluble materials, the lung burden may be predicted using mathematical models based on experimental studies of pulmonary deposition and clearance for individual laboratory animal species and aerosol sizes (Raabe *et al.*, 1977; Raabe *et al.*, 1988; Snipes *et al.*, 1983; Snipes 1986). In addition, prechronic testing can supply both deposition and retention data. Prudency dictates that the prechronic lung burden data be checked to ensure the accuracy of the models. Collectively, these methods provide a reasonable capability to the investigator for estimating the outcome (lung burden) of exposure levels chosen for a chronic (2-year) study.

For a steady-state lung burden concentration of b, e.g., 20 mg particulate material/g lung tissue (Chan *et al.*, 1984; Lee *et al.*, 1986; Muhle *et al.*, 1988; Wolff *et al.*, 1987; Snipes, 1989), the maximum aerosol exposure concentration could be estimated (with proper dimensional corrections) from the equation

maximum aerosol concentration

$$= \frac{b}{f} \times \ln_e 2 \times \frac{\text{lung mass}}{T\frac{1}{2}} \times \frac{1}{\text{Dep}} \times \frac{1}{\text{MV}},$$

where f is the average fraction of time dedicated to inhalation exposures, $\ln_e 2$ is the natural logarithm of 2, $T\frac{1}{2}$ is the nominal lung retention half-time for the deposited particles, Dep is the fraction of inhaled particles that deposit in the deep lung (pulmonary region) and MV is the normal resting minute volume of respiration. With the possible exception of b which can be assumed to be the same for all species when dose-effects are equated, all of the parameters in the equation are a function of species.

For example, in the case of the rat let $T\frac{1}{2}$ = 100 days or 144,000 min; fractional deposition = 0.068 for 2 μm MMAD aerosol (GSD=2) (Raabe *et al.*, 1987); lung mass = 2 g; b = 20 mg/g; f = 0.1786 (6 hr/day, 5 day/week 24 hr, 7 day-week or 30/168, and minute volume = 0.15 liter/min.

$$C_{\max} = \frac{20 \text{ mg/g}}{0.1786} \times 0.693 \times \frac{2 \text{ g}}{1.44 \times 10^5 \text{ min}} \times \frac{1}{0.068} \times \frac{1}{0.15 \text{ liter/min}}$$

$$C_{\max} = \frac{27.72 \text{ mg}}{0.026 \times 10^4 \text{ liter}}$$

$$= 1066 \text{ mg}/10^4 \text{ liter or } 106.6 \text{ mg/m}^3$$

Thus, to achieve a steady-state lung concentration of 20 mg/g in the rat requires an insoluble aerosol concentration of 106.6 mg/m^3. It should be noted that the calculation of a high steady-state lung burden (>2–5 mg particle/g lung) will almost always lead to a reduction in particle clearance and a change in lung buildup that comes closer and closer to unity. Pragmatically, the steady-state model will overestimate both the time and test material needed. Thus, the actual lung burden will be in excess of the computed steady-state burden. In addition to this example, Morrow has proposed other models for exposure level estimations (Morrow, 1986; Morrow and Mermelstein, 1988).

For inhalation toxicity evaluations with rodents, the test aerosols should typically have a MMAD of 3 μm or less with a geometric standard deviation of no greater than three to maximize alveolar deposition. Aerosols with even a smaller MMAD of 1 to 2 μm and a tighter size distribution, i.e., a geometric standard deviation of less than two, would be preferable. However, this is sometimes not possible due to the nature of the material required in large quantity for chronic toxicologic study. For hygroscopic aerosols a smaller size, approximately 1 μm MMAD, is preferred recognizing that the particle will enlarge in the humid environment of the respiratory tract. If "enrichment" of small particle sizes is required to increase the respirability of the test dust aerosol, the respective respirable fractions of the test and modified test dust should be measured to reconcile the exposure levels used in chronic toxicity testing with those expected or documented under typical conditions of use. Some materials for which toxicity or carcinogenicity testing evaluations are desired may normally occur as particles with sizes greater than a few micrometers and perhaps as large as several hundred micrometers. Such powders may be physically altered to produce aerosols with MMAD's of a few micrometers to facilitate the experimental study of their toxicity. Since such approaches increase the deposition, dose, and possibility of effects of the material in relation to those expected under normal conditions, a knowledge of the differences in respirabilities between the enriched and the normal dusts in both the test animal and man is necessitated.

A minimum of two years of exposure is recommended for chronic inhalation studies in both rats and mice. In the case of rats, they could be held for an additional 6 months without exposure prior to the scheduled kill or until 20% survival is reached for the purpose of maximizing incidences of tumors. The primary basis for the extended holding period for rats is that results from recent stud-

ies with Fischer-344 rats exposed to a range of concentrations of diesel exhaust demonstrated an excess of tumors only at the highest exposure concentrations and near the end of the rat's life span (Mauderly *et al.*, 1987). The vast majority (approximately 80%) of the exposure-related lung tumors were observed in rats killed or that died after 24 months from the initiation of the 5-day per-week exposures. These results emphasize the value of observations beyond 2 years for detecting pulmonary carcinogenesis. However, extension of the duration of an inhalation toxicology study might not be appropriate/possible for all chemicals or with all strains of rats due to variations in life spans or for other species commonly used in chronic inhalation toxicity studies, e.g., mice and hamster, whose life spans do not permit a 24-month exposure duration and postexposure follow-up.

A related question is the exposure duration that is warranted if observations of lifespan shortening diseases such as pulmonary fibrosis or other indications of chronic toxicity are made in addition to, or in lieu of, tumorigenicity. If tumors are detected between the 15th and 20th month of exposure, one approach might be to maintain the rats for 30 months following initiation of exposure or until a 20% survival is reached. Alternatively, during the post 24-month exposure period animals could be killed periodically to show either progression of the lesion or regenerative repair, since one or the other usually occurs. It is implicit that if life span shortening occurs, it is important that adjustments be made to ensure that useful histopathologic data are obtained.

A single set of exposure levels cannot be recommended that will be applicable to all chronic inhalation studies. For aerosols not causing impairment of clearance in a 90-day study, one must look for a lack of proportionality between lung burdens and exposure concentrations over the range of concentrations of the test aerosol. Such observations can be made by plotting lung burden versus days of exposure and noting changes in slope to detect increased lung burden of the test aerosol or by constructing a matrix using air concentration (exposure level) and duration of exposure, e.g., 30, 60, and 90 days, in which one enters the ratio of the lung burden to the exposure level for each box denoting a specific time period and exposure level. The higher the ratio for an exposure level, the more disproportionate is the lung burden at one or more durations of exposure. In prechronic studies, when animals that are killed lung burden data should be obtained. These determinations should be coordinated timewise with other serial kills programmed for determination of toxicologic endpoints. The lung burdens can be obtained by determining the mass of test material per gram of lung by chemical or other appropriate means. These results may then be compared with model predictions to determine if lung burdens in excess of those predicted from kinetics at lower levels and overwhelming of clearance mechanisms are occurring. Within a given study, the relationship between lung burden and exposure concentration can be compared for the several exposure concentrations to give an indication of altered kinetics at the higher exposure levels relative to the lower levels (Wolff *et al.*, 1987).

Depending on the physical/toxic properties of the test aerosol, both 14-day and 3-month studies can provide varying degrees of useful toxicologic information for planning and interpreting chronic exposure studies. Such studies also provide the opportunity for rehearsing all methods and fully evaluating aerosol generation and characterization techniques before initiating the more expensive chronic studies. In many cases, results from as many as five exposure levels plus control for both 14- and 90-day studies are valuable in selecting exposure levels for the chronic study. A range of exposure concentrations should be selected to assure inclusion of one level that potentially suppresses pulmonary clearance and results in pathology in addition to one lower level which is free of these effects.

Testing should not be done at the highest exposure concentrations that are technologically feasible. The exposure concentrations should be selected so they produce an optimal range of lung burdens. With exposure to aerosols of MMAD of less than 3.0 μm and a geometric standard deviation of less than 3.0, acute studies using aerosol concentrations of greater than 1,000 mg/m^3 or long-term studies using aerosol concentrations in excess of 100 mg/m^3 are of limited utility in assessing human health posed by inhalation of aerosols unless it is known that human exposures at or near these levels actually occur.

DISCUSSION

In the course of conducting inhalation toxicology studies, one must consider issues specific to administration of the test material by inhalation as compared to those of other routes of administration. Exposure by the latter means is often a case of administering a specific quantity of the test chemical whereby the dose to the target organ(s) is rapidly achieved. Conversely, when insoluble materials are aerosolized, it is much more difficult to quantify the dose of the test substance and steady-state lung burden may take months to achieve or may never be achievable as in the case where "critical masses" of the test substances in the lung markedly impair pulmonary clearance mechanisms. Thus, there are important biologic differences which distinguish establishing maximum aerosol concentrations in inhalation studies from maximum tolerated doses using the oral and parenteral means of administering varying amounts of a test chemical to experimental subjects.

This paper has attempted to highlight the importance of understanding the relationships among exposure concentrations and durations, the particle size and species dependency of fractional deposition of an aerosol in various regions of the respiratory tract, dose (lung burden), and toxicologic response as they relate to inhalation toxicology studies. Cogent details on these critical issues were provided as well as guidelines and recommendations for conducting contemporary inhalation toxicologic and carcinogenic studies.

Researchers who conduct inhalation toxicology studies must be cognizant that the topic of "Maximal Aerosol Exposure Concentrations in Inhalation Studies" is a rapidly developing area in terms of knowledge. Accordingly, the contents of this article are intended as guidelines and not as absolute rules. The key point that is made is that all toxicity studies conducted with inhaled particulate material must involve a determination of the actual amount of test material in the lung as a function of time. The measurement of lung burden is appropriate at all exposure levels at 3, 6, 12, 18, 24 and 30 months after initiation of exposure in a chronic bioassay. Temporal determinations of lung aerosol retention must also be incorporated into the prechronic studies to adequately plan a chronic bioassay.

For additional information on this complex topic, the reader is also referred to other papers on this subject (Morrow, 1986; Morrow and Mermelstein, 1988; Snipes, 1989).

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