Optimizing a Test Bed System to Assess Human Respiratory Safety After Exposure to Chemical and Particle Aerosolization

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Abstract

In the last decade, there has been a movement within environmental health and toxicology research to progressively phase out animal testing and implement more rigorous and validated cell culture models. This movement is checked against the rapidly evolving field of immunotoxicology and measurement of immune-mediated cellular responses. Both trends are driven by many variables, some of which are common in both (i.e., increased time constraints; social, financial, and regulatory pressures; and general need to develop modern test bed systems for safety-by-design product development). The need for non-animal alternative test methodology is especially important in the field of immunotoxicology, where increased emphasis has been placed on distinguishing dermal, respiratory, and gastrointestinal sensitization and/or irritation, defining key markers for hazard identification, and developing potential high-throughput screening processes. With these drivers in mind, this article summarizes the key components of developing a prototype test bed system to rapidly determine immunotoxicological responses following exposure to aerosolized materials. Inhalation exposure to aerosolized substances (e.g., chemicals and particles) is a common occurrence for individuals working in research laboratories, engaged in industrial processes, in consumer use of household products, and during the waste disposal process. We recommend research priorities with emphasis on developing continuous flow testing systems, inclusive of aerosolization, cell culture exposure, and immunological endpoint analyses.

Keywords: aerosolized materials, air–liquid interface, hazard identification, immunotoxicology, *in vitro* toxicology, pulmonary toxicity

Introduction

RESPIRATORY EXPOSURE to airborne chemicals and other materials can lead to many adverse health effects, including asthma, rhinitis, and reactive airways dysfunction syndrome.¹ Standardized and well-validated non-animal alternative methods and models for identifying hazards, measuring exposure, assessing risk, and classifying particles, vapors, fumes, or liquid droplets are currently underdeveloped and require additional improvements.^{2–5} There are increasing pressures from regulatory bodies across the globe to develop rapid screening methods to assess the potential adverse human health effects after exposure to aerosolized substances, for example, chemicals and particles. Recent directives from the European Regulation on Registration, Evaluation, Authorization, and Restriction of Chemicals (REACH) and new initiatives from the U.S. Environmental Protection Agency (EPA) fuel the critical need to develop *in vitro*-based

immunotoxicological test beds for single-component products (such as solvents) as well as combinatorial products (such as nano-enabled drug products).^{2,6–9}

This article presents a prototype test bed system designed to optimize the input parameters and quantify the resultant data generated after exposing human lung cocultures to aerosolized chemicals and particles. The model uses a gently impacting aerosol generating system and cell cultured at an air–liquid interface (ALI) that are interchangeable and maintained in a physiologically relevant environment for adjustable periods of time. Two notable critical factors integrated within its design are as follows: (1) quantifiable aerosol size and mass concentration and (2) multiplexed inflammatory response indices. Both these features allow for identification of or distinction among respiratory irritation, sensitization, or allergic reactions.

The following discussion outlines some of the most important considerations when designing an *in vitro* pulmonary

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toxicology platform for exposure of aerosolized substances to lung cell cultures. For example, the screening of potential formulations of inhalable pharmaceuticals, fragrance materials, engineered nanomaterials, aerosolized pathogens, aerosolized probiotics, wildfire ash, and other hazardous air pollution could be tested and provide information about toxicology endpoints and inflammatory responses. Among the considerations discussed herein are immunotoxicological effects, pulmonary cell culture conditions, biomechanical motions and processes, aerosol dose metrics and relevant exposure scenarios, and perspectives from regulatory agencies.

Immunotoxicological considerations

An aerosolized substance (such as a particle, vapor, fume, or liquid droplet) can be labeled as an irritant, sensitizer, or allergen. These chemical-induced adverse effects are generally classified under the broad category of inflammatory responses. The specific inflammatory response can either be immune-mediated or completely void of inducing any immune-oriented cytokines or cells. Either way, the response is based on mammalian model endpoints and the deduced cellular mechanisms of action identified following exposure (Fig. 1).

FIG. 1. Biomechanical and immunotoxicological considerations. (A) Illustration shows the physiological location of the ALI within the human respiratory system. To the left, the upper conductive zone includes nasal cavity, mouth, pharynx, and trachea. The lower respiratory zone includes bronchi, bronchioles, and alveolus. The primary ALI is located within the alveolar space. (B) Illustration shows two different pathways that a chemical/particle can induce after pulmonary exposure. Pathway A follows the induced adverse response of a nonsensitizing, nonallergenic irritant, while Pathway B shows the induced adverse response of a sensitizer. ALI, air–liquid interface.

More specifically, in an effort to distinguish the mechanisms responsible for irritation effects from those induced by sensitizers/allergens, it is important to define the impact of solid particulate matter versus liquid biochemical materials. Exposure to solid substances have been shown to trigger either a “physical,” or mechanical adverse effect to tissues (such as abrasions or shear stress), or a “biochemical” adverse effect to cells may induce specific gene expression and/or mediator production pathways (such as promotion of methylation or induction of oxidation response signaling).^{10–12} When a substance provokes a nonimmunological inflammatory response (such as abrasion or dryness), it is conventionally termed an irritant. When exposure to the substance results in an immune-mediated inflammatory response (such as redness/erythema or itching), it is labeled as a potential sensitizer.¹³

The distinction between dermal irritants and sensitizers is well defined by the U.S. Food and Drug Administration (FDA)¹⁴; however, such a clear distinction has not been drawn for substances exposed to the respiratory system. Respiratory sensitizers and irritants can result in physical symptoms such as coughing, wheezing, or tightness in the chest.¹³ In general, these health effects are readily reversible when the irritant stimulus is removed. In contrast, a sensitizer is ascribed to a systemic adaptive immune response, which often causes an irreversible physiological change that can be reactivated upon subsequent exposure to the sensitizing material.⁵ Due to the overlap in outward symptoms, respiratory sensitizers and irritants can only be distinguished by assessing immunological responses, such as through cytokine fingerprinting.¹⁵ Such a distinction is important for three reasons. First, to determine the effects of aerosolized substances on immune function. Second, to determine when additional immunotoxicity studies should be conducted. Third, to determine when additional mechanistic information could be identified to help characterize the significance of an effect on the immune system.¹⁴ These three reasons can be addressed by developing and utilizing an *in vitro*-based test bed system designed to rapidly determine immunotoxicological responses after exposure to aerosolized substances.

Cell culture considerations

In vitro and *in vivo* models have been criticized for their lack of reproducibility and lack of correlation to each other.^{16,17} *In vitro* models have additional disadvantages, such as being too simplistic of a representation of the complex human pulmonary system.¹⁶ Despite these disadvantages, *in vitro* models have advantages such as ease in experimental procedures, rapid data collection, and cost effective high-throughput screening capability. In some cases, the use of *in vitro* models is warranted before proceeding with an *in vivo* study. This type of design potentially affords faster determination of proper dosing range, elucidation of probable mechanisms of toxic action, and refinement of exposure and endpoint analysis techniques.¹⁶ With further research into improved *in vitro* models, such as multicellular cocultures and three-dimensional (3D) models, the disadvantages of *in vitro* models are becoming less pronounced and more physiologically relevant alternatives to animal models are a viable reality (Fig. 1).

Pulmonary cell considerations

The lung is a highly complex organ with multiple cell types, elongated cavities, highly dense pores, and unique

functionality. The complexity is heightened along its central axis; functionality of the respiratory tract cell types change from the nasopharyngeal cavity to the bronchi, bronchioles, and, ultimately, the alveolar–capillary unit. The porous regions (a.k.a. alveoli) are especially vulnerable to particulate and/or liquid aerosol exposure and have been an important component for pulmonary toxicological evaluation.^{18,19}

The alveoli compose 99% of the surface area of the lung and are the primary cell type responsible for gas exchange at the alveolar–capillary interface.²⁰ In the absence of aerosol exposure, the main function of this lung space is O₂ to CO₂ exchange. However, after exposure to aerosolized substances, the alveoli works with the respiratory tract to initiate a multifaceted approach for particle clearance mechanisms: a mucociliary escalator in the tracheal and bronchiolar epithelium, a surfactant and thin tissue layer separating aerosol from the blood vessel compartment at the alveolar level, and phagocytosis at the individual cell level.^{20–23}

The alveolar space is composed of three main cell types: alveolar cells that are epithelial cells responsible for gas exchange and produce lung surfactant; alveolar macrophages that are immune cells resident to the lung parenchyma; and dendritic cells that function, when recruited via immune cellular signaling, as antigen-presenting cells (APCs). APCs are required for propagating the inflammatory signaling needed for downstream cytokine production in the adaptive immune response associated with the allergic phenotype in a manner similar to Langerhans cells for dermal sensitization and allergy.^{24–26} The cross talk that occurs between all cell types appears to be necessary for activation of the dendritic cells (Fig. 3). This knowledge, taken collectively with an understanding of the types of clearance mechanisms afforded in the alveolar space, becomes a vital aspect for building an *in vitro* inhalation safety assessment and efficacy test system. Therefore, when designing cell-based toxicological model systems, inclusion of the three cell types defined is imperative for accurate representation of the complex activity in the lung for proper *in vitro* study and assessment (Fig. 2).

Recent toxicological studies have incorporated Type II epithelial cells (such as BEAS2B or A549) and monocytes (such as U937 or MDM), which can differentiate into macrophages in cocultures.^{16,27–30} These cell lines are ideal for *in vitro* toxicological studies as the macrophages directly respond to foreign materials and the Type II cells respond to damage during exposure.¹⁶ In addition, macrophages secrete a variety of cytokines constitutively, or in response to stimuli, which can then be assayed for postexposure with the antigen presenting dendritic cells.³¹ The cell line options for dendritic cells are less mature. Some cocultures have included dendritic cell lines (such as MDDC or JAWSII) or primary cells isolated from blood.^{27,28}

Three-cell cultures are not only more representative of *in vivo* tissue composition, but (when cultured on membrane inserts) are 3D, allowing for *in vivo*-like arrangement.^{28,30} In the 3D, three-cell coculture model, Type II epithelial cells are grown on a porous membrane, macrophages are seeded on top of those cells, and dendritic cells are suspended in media underneath the membrane. When the cells are compared to *in vivo* tissues, the multicellular system has been found to be morphologically and quantitatively (based on cell type ratios) representative of the rodent model.^{27,31} Advanced and more complex *in vitro* systems, such as 3D

FIG. 2. Coculture assembly for three-cell 3D *in vitro* pulmonary model. (A) Monolayer assembly of pulmonary cells into a coculture. First, human lung airway epithelial cells (ECs) are grown in a monolayer on porous membrane, gelatinous protein mixture, or collagen. Second, human alveolar macrophages (AMs) are added to the EC monolayer. Third, the coculture (CC) is placed in aerosol exposure chamber where aerosols are characterized continuously in real-time. After exposure, CC is removed from aerosol exposure chamber and the porous membrane is laid above dendritic cells (DCs) to observe activation and cytokine expression. (B) Phase contract microscopy image of ECs. (C) Phase contract microscopy image of AMs. (D) Phase contract microscopy image of DCs. 3D, three-dimensional.

coculture models, provide a step toward better alternatives to animal testing systems by allowing the study of not only single cell type interactions to foreign materials but also the study of the interactions of different cell types with one another (Fig. 3).

Biomechanical considerations

To further increase physiological relevance, attempts to mimic real-world exposure scenarios have been made. For example, in relationship to inhalation studies, a strong preference has been expressed in the toxicology community to focus exposure of lung cells at the ALI rather than submerged cells.^{32–34} This exposure system more closely resembles physiological conditions in the lungs; allows unaltered, uniform, and dosimetrically accurate delivery of aerosolized materials; and eliminates the potential for media to interfere with material behavior or cellular response (Fig. 4).

Conventional exposure, involving suspension of particles in liquid medium, has limitations for exposure concentrations and may alter the particle physiochemical properties. For example, suspending particles in media and exposing submerged cells has been shown to promote particle agglomeration.²⁸ This agglomeration, beyond increasing particle size, could cause the particles to settle onto the cell layer at a faster rate than individually suspended or aerosolized particles, potentially influencing biological interactions. One study showed aerosolized particles exposed at the ALI

of the 3D lung cell model to undergo less agglomeration and thus less sedimentation compared to previously published submerged cultures.²⁸ Direct deposition of the particles at the ALI not only minimizes physicochemical changes to the particles but it also mimics particle deposition after inhalation in the respiratory tract. More realistic particle deposition will potentially lead to a more physiologically representative cellular response when compared to submerged cell exposure experiments.

Understanding the potential adverse effects on the immune system after exposure to aerosolized substances is an important component of the overall evaluation of chemical toxicity. Evidence of immunotoxicity, such as morphological cellular changes due to inflammation, usually can be observed in standard nonclinical toxicology studies using either *in vivo* (animal) or *in vitro* (cell-based) models. *In vitro* models, however, are used to give preliminary indication of upstream immune system effects and, therefore, often suggest whether in-depth studies should be considered. ALI *in vitro* coculture model systems designs should become the standard when studying the human health effects of aerosols. There are many routinely used substances that are intended to be exposed to the consumer population. Similar to all potential toxicants in breathing air, these routinely used substances induce potential sensitization effects and may interact with other foreign substances in the body to form metabolites in tissues. Ultimately, it is the dose that determines the potency potential.^{35–37}

Aerosol exposure considerations

Exposure to aerosols is generally accomplished using an impactor (Fig. 4). An aerosol impactor uses a combination of gravitational settling and forced airflow of aerosolized substances as a delivery mechanism to cells in culture. Ideally, this type of “gentle” impaction operates in a batch-type manner and would consist of a biocompatible cylinder (such as Teflon or stainless steel), which is mounted and sealed to prevent particle loss. The choice of cylinder material is also a critical factor in controlling specific or non-specific aerosol binding. The use of Teflon or stainless steel for the chamber construction material, combined with the use of an aerosolize neutralizer prevents liquid or solid aerosols from sticking to the chamber walls. The entire apparatus should be oriented with the inlet at the top and aerosol deposition at the bottom. The base should contain a sliding tray (to function as the point of entry for cells grown in Petri dishes or well plates) and a sampling port (for real-time aerosol size and mass concentration measurements via an Aerodynamic Particle Sizer). The entire system should be housed in an aseptic workspace with temperature and relative humidity controlled to 30°C and 75%, respectively.³⁸ Aerosols ought to be tested and confirmed for homogeneous monodisperse deposition across the sliding tray (where the cell culture resides). The chemical/particle exposure dose can then be measured quantitatively by mass using quartz crystal microbalance or qualitatively by particle number using confocal microscopy.^{39,40}

Aerosol size and mass concentration can be controlled by a N₂ gas dilution. Target mass concentrations are variable over a wide dosing regimen. Ideal dosing concentrations should be comparable to that expected from aerosolized occupational or consumer based scenarios.⁴¹ To achieve target size and mass concentrations, the following physico-chemical properties should be included in the study: chemical composition, physical appearance, melting point, boiling point, vapor pressure, solubility in water, and specific gravity.^{42–45}

FIG. 4. Aerosol exposure to cells in culture. (A) Schematic representation of an aerosol exposure chamber that houses aerosol flow inlet, void space for particle movement, gravitational settling chamber, and housing for pulmonary cell cocultures. Larger particles (>3 mm) have high velocity and are removed from the exposure chamber before entering the gravitational settling chamber. Smaller particles (<3 μm) pass through the aerosol flow inlet and are exposed to cells. Results collected from exposure to 1 and 3 μm-sized particulates are ideally suited for this apparatus and are optimally sized to compare to results collected after particle inhalation using rat *in vivo* model. (B) Photograph of the aerosol exposure chamber intact. (C) Photograph of the cell culture dish insert located at the bottom of the chamber.

There are approximately five developed aerosol delivery technologies described in the literature.⁴⁶⁻⁵³ A few of them are also commercially available. Impaction by Vitrocell Aerosol System has an aerosol size range of 2 to 6 μm . It is temperature maintained, uses a reservoir for humidity control, and contains multiple chambers for varying exposures.^{14,54,55} Diffusion/Impaction by NaviCyte System has an aerosol size range of <1 μm . Similar to the Vitrocell model, it is also temperature maintained, uses a reservoir for humidity control, and contains multiple chambers for varying exposures.^{56,57} The ALICE Gravitational Settling has an aerosol size range of 100 to 250 nm. It uses in-line humidity control and has shown to deposit particles uniformly across the cell culture population.⁵⁸ The electrostatic precipitation system has an aerosol size range of 20 nm to 3 μm and has a high collection efficacy.^{59,60}

An important point of consideration in the conduct of any inhalation exposure study, whether it be *in vivo*, *in vitro*, or *in silico*, is to establish an understanding of the dose metrics and how the dose delivered aligns across the study types for appropriate comparison. An *in silico* model, such as the Multiple Path Particle Dosimetry Model (MPPD) is a well-established, validated, and accepted model that can help identify the location of a particular droplet/particle size is expected to reach and deposit and where lesions of cellular damage may occur.⁶¹⁻⁶⁵ A mass per unit surface area can then be applied to both *in vivo* (which has been done successfully for validation purposes) and *in vitro* applications for establishing the appropriate droplet/particle size to emulate the intended exposure scenario that investigators wish to assess for potential exposure effects.

In addition to the exposure scenarios described above, a minimum of two types of controls should be included; first, cells that are never placed in the gentle impactor and remained submerged in media are considered the baseline control; second, cells placed in the chamber and removed from the media, but do not undergo exposure, are regarded as the negative control.

Regulatory considerations

There is a need for biologists and toxicologists to team with aerosol scientists to assess the potential risk of inhaled

solid particles and liquid droplets. The aerosol community has the tools and expertise to develop and perform ALI *in vitro* exposure systems; meanwhile, the toxicology community is able to determine and carry out the proper assessment of the biological impact of the consumer and/or occupational aerosolized products.⁶⁶ Together, these teams can strengthen the product pipeline by ensuring safety and limiting toxic human health exposures. *In vitro* sciences offer this advantage in a cost-effective and high-throughput manner.^{14,60,67-71}

Respiratory exposure to airborne substances classified as irritants, sensitizers, or allergens is not only relevant for potential consumer exposure but also for occupational health scenarios. There is little distinction between respiratory irritants versus sensitizers with regard to hazard identification and risk assessment despite the differences in biological responses.

Since inhalation is an important exposure route for a wide range of aerosolized products, test systems that simulate inhalation exposures are needed to screen for biological effects in a rapid, reliable, reproducible and cost-effective manner. The minimum requirements for a system, such as the one described in this article, should be capable of generating aerosols for a variety of particles, vapors, fumes, or liquid droplets over increasing dosing concentrations integrated with a cell culture model maintained in an ALI.^{14,60,67-71} The recommended system would produce a range of exposure levels by controlling the aerosol input and the exposure duration. Aerosol size distributions from the impactor would also reveal an increase in mass deposited on the cell culture as a function of exposure time and surface area. This is vital to the ideal operation of an *in vitro* exposure system. Exposure duration and relative deposition will dictate the magnitude of any potential effect that will be assessed in the system. In addition, as the cell types vary at different locations along the respiratory tract, different effects may be anticipated. An understanding of the regulatory limits for occupational exposure and/or product formulations, based on specific observed effects, will help to guide the operational definitions of the exposure system.

Specific to respiratory sensitizers, Table 1 describes the generally accepted definitions from a variety of different agencies and organizations.

TABLE 1. DEFINITIONS OF RESPIRATORY SENSITIZERS FROM INTERNATIONAL AGENCIES AND ORGANIZATIONS*

Agency	Definition	Website
OSHA's Hazard Communication Standard	A chemical that causes a substantial proportion of exposed people or animals to develop an allergic reaction in normal tissue after repeated exposure to the chemical	https://www.osha.gov/dsg/hazcom
OSHA's Globally Harmonized System of Classification and Labeling of Chemicals	A substance that induces hypersensitivity of the airways following inhalation of the substance	https://www.osha.gov/dsg/hazcom/ghs.html
Canadian Centre for Occupational Health & Safety	Substance with the potential to act, through whatever mechanism, to create a situation of airway hypersensitivity where none previously existed	www.ccohs.ca
United States Army	A substance that induces hypersensitivity of the airways following inhalation of the substance	www.dod.org
European Environmental Bureau	A substance with the potential to act, through whatever mechanism, to create a situation of airway hypersensitivity	www.istas.net/risctox/en/index.asp?idpagina=612

*References.^{5,66,67}

Recommendations

This article presents a conceptual model of a gently impacting aerosol generating system. This system is able to expose cell cultures at an ALI with a desired aerosol size and mass concentration. The cells exposed at the ALI are interchangeable and maintained in a healthy environment for adjustable periods of time. We recommend a three-cell combination, representative of the three primary cell types present in the alveolar space, arranged as a 3D coculture system. Postexposure comparison of the inflammatory response of the coculture system should allow for respiratory irritant, sensitizer, or allergen identification/distinction. Previously, exposure at the ALI has shown to reveal differential cytotoxic and immune responses. This framework for integrating aerosol engineering with *in vitro* toxicology lays a path to differentiate respiratory sensitizers from irritants using a high-throughput, cost-effective, cell culture-based model system. Such a model is needed to develop standardized methods for classifying inhaled substances, identifying hazards, measuring exposure, and assessing risks.

Next generation exposure systems should be designed as a more cost-effective screening tool aimed to simulate inhalation exposure in a dose-dependent manner, when compared with other conventional indirect or liquid-liquid cell culture exposure approaches. The system should be flexible/adaptable, modular, compact, inexpensive, and capable of integration with different aerosol generation apparatus appropriate for solids, liquids, or gases. In-line humidity control, gravitational settling, and gentle airflow impaction can be incorporated into the engineered system to deliver aerosols to cells in culture and provide size-dependent deposition efficiency data. The cylindrical design allows for exposure of large cell populations that facilitates the collection of micrograms amounts of cell tissue homogenate for multiple endpoint analyses. Arguably the most important characteristic of this system is its ability to aerosolize the native material without any solvent or other diluent, thus allowing for streamlined analyses of data without confounding experimental conditions.

The test bed system described in this article is designed to assess human respiratory safety after exposure to chemical and particle aerosolization. With the increase in effort to replace, reduce, and refine methods for more ethical use of animals in testing, aerosol exposure *in vitro* systems should continue to be developed and optimized.⁷² Our system not only presents aerosols to cells cultured at the ALI but is also embedded with methods to quantitatively measure and control its components. By the time an aerosolized chemical or particle reaches the alveolar space, the cells are exposed to aerosols in a laminar flow.^{73,74} Therefore, with the addition of gentle impaction, this system is designed to simulate the type of flow experienced at the ALI in an *in vivo* scenario under natural breathing conditions.

Conclusions

In this article, we discuss the development of a gentle impactor aerosol exposure apparatus for air-liquid *in vitro* cell culture-based exposure. The system works efficiently for short-term exposures at a variety of doses by controlling concentration and exposure times. With additional adaptation,

long-term exposures may also be possible. The engineered system has several advantages:

1. First, the chamber is simplified and has less moving parts when compared with other impaction units
2. Second, the chamber is easier to clean between test materials compared with other models
3. Third, the exposure system can test various liquid and solid materials when integrated with an aerosol generation apparatus that uses various aerosolizing technologies for solid and liquid materials
4. Fourth, a single gentle impactor allows dosing over an entire well plate rather than an array of gentle impactors over each well of a well plate
5. Fifth, the system is able to generate aerosols of the native chemical/material without the use of dilution solvents
6. Sixth, the system is capable of working with commercially available cell systems or a customized coculture system.

This study outlines a critical step in the evolving area of high-throughput screening models that give preliminary indication of potential toxicities associated with airway exposure to aerosolized materials. Specifically, measuring indices that distinguish chemicals/particles as irritants or sensitizers are useful when deciding if a substance warrants further investigation, requires validation, or is a candidate for other bioassays, such as the Direct Peptide Reactivity Assay (DPRA).⁷⁵ The screening model presented here aligns with the Tox21 initiatives of conducting real-time toxicity assessments using a single experiment to measure multiple endpoints.^{76,77}

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