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(1 → 3) β-Glucan induces multimodal toxicity responses in parallel exposures of model human lung epithelial cells and immature macrophage

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Abstract

Many epidemiological studies have associated bioaerosol exposures with a variety of adverse health effects; however, the role of bioaerosol components in the development and manifestation of hypersensitivity and non-infectious respiratory diseases remains unclear. Despite many studies which have examined allergic responses to bioaerosols, less is known about non-allergenic effects. In order to elucidate the mechanisms by which bioaerosols can exert non-atopic stresses on a cellular level, there is a need for improving existing in vitro approaches. In response, a cohort of toxicology assays were optimized to create a robust analytical suite for studying the effects that biogenic atmospheric pollutants generate on two model human lung cell lines (A549 epithelial line and GDM-1 immature macrophage line). To demonstrate the utility for studying the cellular responses to select bioaerosols, cells exposed to curdlan (a linear (1 → 3)-β-glucan) were examined in a composite cytometry platform. Results suggest that curdlan has the potential to elicit significant responses in A549 and GDM-1 in two or more toxicological modes associated with exposure to airborne particulate matter. As designed, this suite provided a more powerful tool for characterizing curdlan-induced toxicological potential than any individual assay. Responses to curdlan were distinctly modal and cell line dependent, suggesting that the use of a suite of toxicological assays, in a common platform on different cell lines, can help provide important insights into the formative toxigenic responses that primary bioaerosols can induce in respiratory cells.

Keywords Airborne particulate matter · Bioaerosol · Curdlan · Toxicity · In vitro

Introduction

Airborne particulate matter (PM) comprised in part, or whole, of biogenic materials is often termed bioaerosols. As a class, bioaerosols include a wide range of biogenic particles including microorganisms (regardless of viability), their fractions, metabolites, and other particles originating from non-microbial life forms, including plant debris and pollen (Fabian et al. 2004). Elevated bioaerosol levels are commonly encountered in water-damaged indoor environments, as well as, agriculture, biotechnological industries, waste management, and health care settings (Douwes et al. 2003). Bioaerosol exposure has been correlated with four major types of adverse health effects: allergy and hypersensitivity diseases, infection, irritation, and toxicity (Fung and Hughson 2003; Madureira et al. 2018). While many studies have focused on allergic responses to bioaerosols, non-allergenic responses have received relatively little attention (Bardana 2003). There is a need for additional toxicological studies, which examine non-allergenic responses in animal models or cell lines, to identify potentially hazardous types/sources of bioaerosols and the cellular mechanisms that underlie negative biological effects.

Existing in vitro research on the respiratory toxicity of bioaerosols has typically utilized a single cell line, most commonly mouse macrophage, and focused on the production or expression of selected cytokines and chemokines (Schwarze et al. 2013). The release of these signaling biochemicals is an important part of the innate immune response and is typically associated with infection, inflammation, or trauma; however, they do not indicate the nature of the cellular stress that has led to their release, nor does their induction indicate specific toxicological endpoints (e.g., DNA damage). In this context, isolating toxicity modes through inference of select cytokine

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induction (e.g., interleukins) can potentially neglect important toxicity mechanisms and limit recognition of conglomerate respiratory responses. The mechanisms by which bioaerosols exert adverse health effects cannot fully be understood without more comprehensive toxicological approaches. These approaches should be complimentary to cytokine observations and have the ability to provide evidence for mechanistic toxicological endpoints and clarification of toxicity pathways (Crook and Burton 2010). In response to this need, a suite of toxicological assays was utilized for the analysis of bioaerosols and other important airborne PM of concern (Turner et al. 2014). We report here findings of the differential effects that (1 → 3)-β-glucan, a ubiquitous aerobiological agent, can have on model human cell lines.

Curdlan, a purified (1 → 3)-β-glucan, was selected as a model bioaerosol fraction for this research. Curdlan is part of a large group of environmentally relevant glucose polymers, which includes a wide variety of structures, and is often generically referred to as *glucan* (Goodridge et al. 2009). This biochemical class includes celluloses, (1 → 4)-β-glycosidic polymers, and (1 → 6)-β-glycosidic polymers, as well as many other short, long, branched, or unbranched α or β isomers, which can occur in varying solubilities, as well as gelatinous hydrates (hydrogels) (Kankkunen et al. 2010). Differences in linkages and chemical structure have been shown to be factors for solubility as well as biological activity (Kataoka et al. 2002). (1 → 3)-β-glucans are non-allergenic, relatively insoluble components found in most fungi, some bacteria, most higher plants, and many lesser evolved plants (Kataoka et al. 2002). (1 → 3)-β-Glucans are structural components of cell walls and comprise an estimated 60% of the dry weight of fungi. Research suggests that their presence is largely independent of growth conditions (Douwes 2005). (1 → 3)-β-Glucans are thought to be one of the most commonly occurring glucan biopolymers and have been associated with negative biological effects; therefore, they have commonly been chosen as a model bioaerosol component for environmental health research (Beijer et al. 2002). (1 → 3)-β-Glucan concentrations, both airborne and surface associated, have also been proposed as a marker for assessing risk in indoor environments (Rylander 2010). Because many bioaerosols can induce biological activity in a variety of hosts, regardless of their own viability, researchers have proposed that (1 → 3)-β-glucan can potentially serve as a useful surrogate to estimate biomass levels in a wide range of environments including aerosols (Rylander 1994). Common sources of (1 → 3)-β-glucan for research have been purified from yeast (*Saccharomyces cerevisiae*), mushroom (*Sclerotium glucanicum* and others), bacteria (curdlan from *Alcaligenes faecalis*), and seaweed (laminarin from *Laminaria digitata*). Studies have also demonstrated that (1 → 3)-β-glucans may have clinical potential, including enhancement of host-mediated induced resistance to infections and antitumor activity (Williams 1997). Because of these properties, a significant portion of the existing research on (1 → 3)-β-glucans has centered on their potential as a component in anti-cancer drugs (McIntosh et al. 2005).

A limited number of epidemiological studies have focused on (1 → 3)-β-glucan. This paucity is primarily because analytical methods for quantifying (1 → 3)-β-glucan were not commercially available or affordable until the last decade. These studies evaluated a range of health effects in relatively large population studies including lung function, nasal congestion, airway hyper-reactivity, atopy, and upper and lower respiratory symptoms; however, few associations were found. The literature on associations between (1 → 3)-β-glucan exposures and lung function remains tenuous. While Rylander (2010) and Douwes (2005) reported adverse effects on lung function after exposure to (1 → 3)-β-glucan, other studies did not support these findings and some studies reported negative associations (Fung and Hughson 2003). Issues with current sampling and analytical methods include a lack of inter-laboratory comparisons and widely ranging estimates of (1 → 3)-β-glucan from environmental sampling analysis (Rylander 1994). Estimates for (indoor) environmental concentrations of airborne β-glucans have been reported to span a broad range from 19 ng/m^3 in some schools up to 631 μg/m^3 in horse stables (Samadi et al. 2009). The highest levels reported have been associated with the indoor atmospheres of confined animal feeding operations (Lawniczek-Walczyk and Gorny 2010). The uncertainty in (1 → 3)-β-glucan exposure assessments has presented epidemiological challenges, and agreements between various toxicological endpoints across contemporary laboratory-based studies remain tenuous. For example, while both in vitro and in vivo studies have shown that (1 → 3)-β-glucan has the potential to stimulate inflammatory effects, to date, these findings have yet to be supported by human exposure or epidemiological studies (Fung and Hughson 2003).

In vitro studies, predominately those undertaken to explore (1 → 3)-β-glucan's potential as a cancer drug, have shown that (1 → 3)-β-glucan possesses important immune-stimulating properties. Research by Apetrei et al. (2010) and Rylander et al. (2010) showed that (1 → 3)-β-glucan is recognized by human immune systems and immediately associates with immune cells including, but not limited to, macrophages and neutrophils. Curdlan recognition in immune cells is mediated by the membrane-bound Dectin-1 receptor(s), although other receptor families have also been implicated. When activated, this transmembrane signaling protein induces an enzyme cascade which can lead to the production of several inflammatory cytokines and chemokines (Rylander 2010). Kubala et al. (2003), for example, exposed in vitro leukocytes to β-glucans at 100 μg/l for 18 h and subsequently measured IL-8 levels nearly two times higher than controls, and IL-6 levels greater than ten times higher than controls. In their

review, Brown and Gordon describe how these immune-modulating activities are specifically opsonized by glucans, and can lead to autoimmunity and other non-infectious diseases (Brown and Gordon 2003). These findings have improved current understanding of how immune responses to fungal pathogens can develop; however, little is known about the activity of glucans in the context of toxicological modes typically associated with exposure to environmental PM, including but not limited to, fungal and bacterial spores which have aged and fractionate in aerosols. Despite many gaps in this environmental health arena, there is sufficient data to suggest that (1 → 3)-β-glucan has the potential to play a significant role in the development of non-allergenic hypersensitivities and other respiratory diseases (Goodridge et al. 2009).

In an effort to better bridge environmental health observations, a suite of parallel assays was utilized to investigate the effects curdlan can have on human cell lines that model key functions of the respiratory system. The suite was comprised of a panel of assays selected to detect several classic toxicity modes that PM may present (genotoxicity, cytotoxicity, and oxidative damage). These assays were specifically selected for their ability to corroborate findings from other assays in the suite. When examined as a group, the results of the suite deliver converging lines of toxicological evidence for both single-mode and multimodal responses. Consideration of a suite of assay results also improves the probability of identifying cellular responses occurring via multiple pathways and, thereby, improves the potential for recognition of toxic effects that may occur as a result of simultaneous stresses.

To increase the information delivered by the suite, the assays were carried out on two separate human cell lines. Cell lines were selected to model two key functions of the respiratory system. First, the human alveolar epithelial lung cell (A549) was selected as a model of the alveolar lining, which exists at the air-liquid interface in the lungs creating the boundary between the circulatory and respiratory systems. Second, the human phagocytic pre-macrophage monocyte cell line (GDM-1) was selected to represent alveolar macrophage cells. These cells are thought to be the immune system's first line of defense against foreign particulate matter entering the body via the respiratory system. Macrophage cells are capable of engulfing foreign particles in the human lung. The use of an analytical suite to compare stress responses of two functionally disparate respiratory cell types enables a more complete assessment of the cumulative responses of the respiratory system. The A549 and GDM-1 cell lines, in particular, were selected because they are well-studied human cell lines, which are commercially available and relatively inexpensive to culture (Schwarze et al. 2013).

We utilized three complementary assays in our comprehensive analysis of the effects of curdlan. The first assay was used to investigate cell death, including differentiation between death via necrotic and apoptotic pathways (Annexin V assay). Secondly, a cell cycle analysis was carried out in parallel to evaluate impacts on DNA synthesis and cell division (Collins et al. 1997). Changes in the cell cycle distribution were quantified to identify potential types of cellular damage (Nunez 2001). Lastly, the third assay measured the accumulation of reactive oxygen species (ROS) within cells exposed to curdlan. When viewed together, the results of the three-assay suite provide a broad toxicity screen, capable of yielding more comprehensive insights than each assay alone. The suite also provides independent validation of important toxicological endpoints. For example, cell cycle and Annexin V are fundamentally different assays which can both detect apoptosis. We report herein a comprehensive suite of results showing distinct cell responses to a model bioaerosol fraction, curdlan.

Materials and methods

Curdlan and controls

Curdlan was purchased from Sigma-Aldrich (catalog no. C7821) and was prepared based on accepted protocols (Kataoka et al. 2002). Because curdlan is insoluble at neutral pH, selected curdlan masses were solubilized in a solution of 0.3 M NaOH containing 0.5% dimethyl sulfoxide (DMSO). This process is known to destroy any endotoxin present (a potential confounder) and denature glycoside helices to produce the forms of linear glucan which are commonly found in microbial cell walls (Jin et al. 2008). Concentrations of 0.15 M and 0.30 M NaoH, with and without DMSO, were tested for their effectiveness in linearizing curdlan. A solution of 0.30 M NaOH with 0.5% DMSO was selected for treatments based on direct microscopic inspection of the solutions. NaOH-treated curdlan stocks were vortexed for 1 min and sonicated at 37 °C for 60 min before being added to cell growth media. Media pH was then adjusted by the addition of appropriate volumes of HCl. Preliminary tests demonstrated that pH adjustments of the media did not affect exposed cells. In all samples, the final DMSO content was equal to 0.5%.

In addition to each curdlan exposure, a control population (untreated) and a vehicle control population (cells exposed to 0.5% DMSO only) were examined. In all cases, vehicle control populations exhibited no significant differences compared to untreated control populations (data not shown). For simplicity, the data shown as "control" herein are cellular responses of vehicle control populations.

Cell culture

Unless otherwise noted, all assay reagents and cell culture media were acquired from Invitrogen (Carlsbad, CA). The A549 human pulmonary type II epithelial adenocarcinoma

cell line was procured from the American Type Culture Collection (ATCC) and was cultured in Ham's F-12 nutrient mixture with a 1% antibiotic-antimycotic solution which contains fungizone, streptomycin, and penicillin (Life Technologies, 15240-062) and 10% fetal bovine serum (FBS). Cells were cultured in a humidified 5% CO_2 atmosphere at 37 °C. For assays and continuous cell propagation, adherent monolayers at 70–80% confluence (exponential growth phase) were harvested following standard protocols (Eisenbrand et al. 2002) with 0.05% Trypsin. The GDM-1 phagocytic human pre-macrophage monocyte cell line was procured from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig). Cells were cultured in RPMI 1640 nutrient mixture with a 1% antibiotic-antimycotic solution and 10% FBS. Exponential growth phase cells were harvested by centrifugation (5 min, 1500 rotations per minute) with greater than 90% viability, as shown by trypan blue dye exclusion (Carero et al. 2001).

Apoptotic and necrotic cytotoxicity

Apoptosis in cell populations was evaluated by detection of externalized phophatidylserine using AlexaFluor488®-conjugated Annexin V. Staining with propidium iodide (PI) was performed concomitantly to distinguish necrotic cells from early apoptotic cells. Assay results were quantified by a flow cytometric quadrant analysis, which allowed comparison of single-cell interrogations. Fluorophore-specific detectors were selected to distinguish between apoptotic and necrotic cells (Vermes et al. 1995). A549 and GDM-1 cells were seeded into 25-cm^2 culture flasks and exposed to curdlan at concentrations of 100, 300, and 500 μg/ml for 24 h and then washed with PBS and re-suspended in Annexin V buffer following recommendations provided by the manufacturer. Each assay was conducted in triplicate (in separate T25 flasks) and a minimum number of 10,000 cell events were recorded for flow cytometric analysis.

Cell cycle analysis

To determine the distribution of cells within phases of the cell cycle, cells were cultured in a 25-cm^2 flask and exposed to curdlan at concentrations of 100, 300, and 500 μg/ml for 24 h. Cells were then harvested, washed in PBS, and incubated in a hypotonic solution containing propidium iodide, according to the methods of Krishan et al. (Krishan 1975). Cells were incubated at 5 °C, in the dark, for a minimum of 24 h (Nunez 2001) and subsequently subjected to flow cytometric analysis. Determination of cell cycle phase was achieved by fluorescence intensity histograms analysis using FloJo analytical software (Tree Star Inc., Ashland, OR). The Dean-Jett curve-fitting algorithm was also utilized when appropriate. Each assay was performed in triplicate (in separate T25 flasks) and a minimum number of 10,000 cell events were recorded for each analysis.

Accumulation of reactive oxygen species

Intracellular ROS accumulation was measured by utilizing a cell-permeant fluorescent probe, 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA, Invitrogen, D-399). Cell populations were mixed in 10 μM H_2DCFDA and incubated at 37 °C in the dark for 45 min, following published protocols (Landreman et al. 2008). Subsequent to incubation, cells were exposed to curdlan at concentrations of 100, 300, and 500 μg/ml for 150 min. Intracellular ROS accumulation was then evaluated by analysis of log-normalized mean fluorescence intensity (MFI) of an H_2DCFDA fluorophore-specific detector. Each assay was performed in triplicate (in separate T25 flasks) and a minimum number of 10,000 cell events were recorded for each analysis.

Flow cytometric analysis

Cell populations were analyzed using a CyAn ADP Flow Cytometric Analyzer (Beckman Coulter, Brea, CA). Post-analysis of raw data was run using FlowJo software (Treestar, Inc., Ashland, OR).

Statistical analysis

Flow cytometric data were screened for normality and all results are presented as the mean, plus or minus the standard deviation (± SD) from three independent experiments. One-way analysis of variance (ANOVA) and Student's t tests, followed by Tukey's multiple-range test (when appropriate), were used for determination of statistical significance mean differences. Statistical significance was defined as a p value of < 0.05.

Results

Apoptotic and necrotic cytotoxicity

As judged by the Annexin V assay, A549 cells exposed to a range of curdlan concentrations resulted in dose-dependent increases in cell death, with significant differences from controls after exposure to 500 μg/ml curdlan (Fig. 1a). The Annexin V assay also differentiates between the two primary pathways of cell death: necrosis (death as indicated by membrane permeability) and apoptosis (programmed cell death). Statistical analysis indicated that exposure to 500 μg/ml curdlan resulted in significantly higher levels of necrotic cell death in A549 cells when compared with

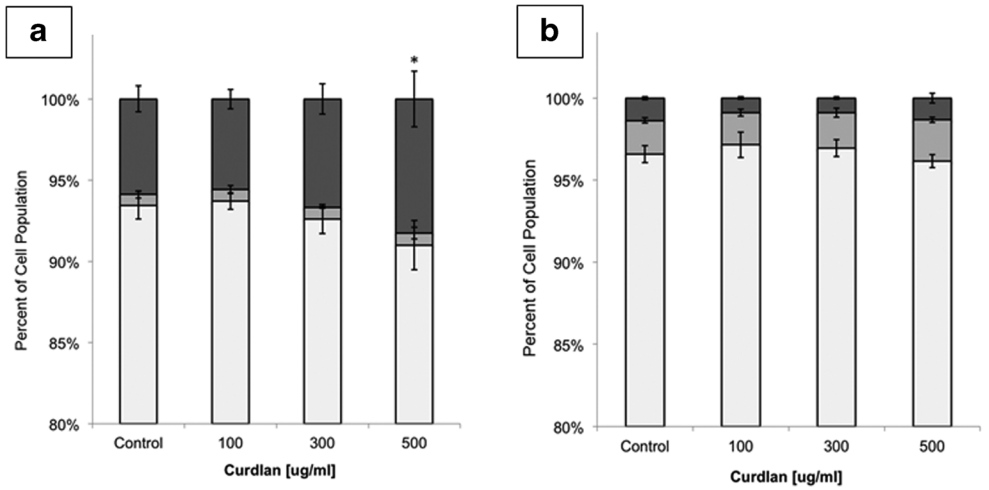

Fig. 1 **a** Flow cytometric analysis of apoptosis and necrosis in A549 cell populations as judged by Annexin and PI staining. Columns represent percent of cell population existing as live (□), apoptotic (▩), or necrotic (■) after 24-h exposure to a range of curdlan concentrations. Error bars represent pooled standard deviation of three independent trials, each of which includes a minimum of 10^4 cell events analyzed. *Significant differences at a 95% confidence level, when compared to controls. **b** Flow cytometric analysis of apoptosis and necrosis in GDM-1 cell populations as judged by Annexin and PI staining. Columns represent percent of cell population existing as live (□), apoptotic (▩), or necrotic (■), after 24-h exposure to a range of curdlan concentrations. Error bars represent pooled standard deviation of three independent trials, each of which includes a minimum of 10^4 cell events analyzed. No significant differences were observed

controls. In contrast, phagocytic GDM-1 cells exposed to increasing concentrations of curdlan did not lead to significant increases in cell death (Fig. 1b).

The results of the two Annexin V cytotoxicity assays suggest that A549 epithelial cells are more susceptible to cytotoxic effects of curdlan than their phagocytic counterparts (GDM-1). Observed differences, however, were just over the detection threshold of the Annexin V assay. Taken together, the results of these assays illustrate that curdlan has the potential to induce different levels of cytotoxic response in respiratory cell lines of differing biological function.

Cell cycle

Cell populations were exposed to curdlan and subsequently examined for changes in the cell cycle phase distribution, when compared to controls. An increase in the percentage of cells in a specific phase of the cell cycle indicates that cells were halted or delayed during this phase and failed to progress toward complete cell division. The identification of an agent's ability to disrupt the cell cycle is an important initial screening for genotoxicity and other cellular defects (Longhin et al. 2013). As illustrated in Fig. 2a, DNA content profiles indicated that A549 cells exposed to curdlan displayed significant alterations to cell cycle distributions. A549 cells exposed to 300 and 500 μg/ml curdlan presented changes when compared to controls, which indicated the accumulation of cells in both the DNA synthesis (S) and Gap 2/Mitotic (G2/M) phases. Accompanying decreases in the portion of cells in the Gap 1 (G1) phase were also observed. Failure of cells to progress through the S and G2/M phases indicates that exposure to curdlan significantly reduced the number of cells able to successfully replicate DNA and undergo successful cell division (Collins et al. 1997).

Analysis of DNA content profiles of phagocytic GDM-1 cells exposed to curdlan also exhibited significant alterations to cell cycle distributions when compared to controls (Fig. 2b). Unlike their epithelial counterparts, however, GMD-1 cells challenged with curdlan resulted in a dose-dependent accumulation of cells predominantly in the S phase. The accumulation of cells halted in the S phase indicates an increase in the number of cells failing to complete DNA synthesis for reasons commonly associated with genotoxicity; however, this can also be caused by other cellular defects (Longhin et al. 2013). Similar to the cytotoxicity results, sensitivity to curdlan was greater in A549 epithelial cells than in their phagocytic counterparts. This finding further supports that functionally distinct respiratory cells have the potential to respond differentially to environmental curdlan exposures.

Detection of ROS

As illustrated in Fig. 3a, A549 cells exposed to curdlan displayed no significant increases in intracellular ROS. Phagocytic GDM-1 cells (Fig. 3b) stimulated by curdlan resulted in a weak induction of dose-dependent increases in intracellular ROS. Significant differences in ROS content were only observed between controls and GDM-1 cells exposed to 500 μg/ml curdlan. Statistical analysis also indicated that cells exposed to 500 μg/ml displayed significantly higher concentrations of ROS than cells exposed to 300 μg/ml.

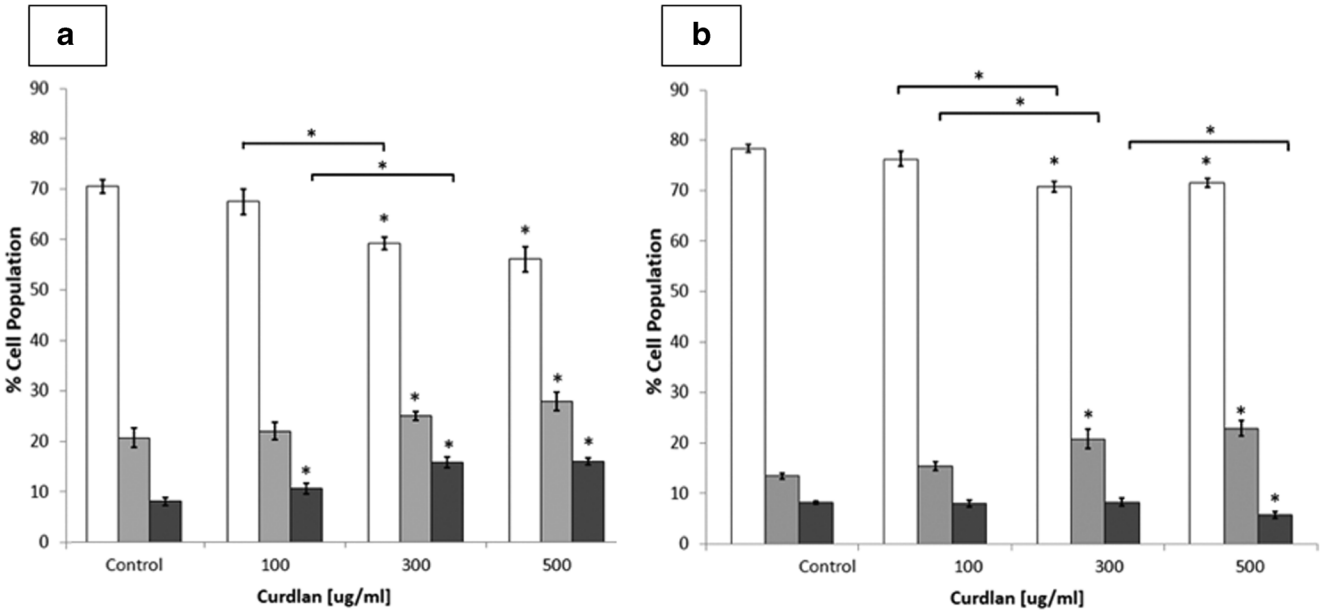

Fig. 2 **a** Flow cytometric analysis of A549 population cell cycle distributions based on DNA content histograms, following 24-h of exposure to curdlan. Columns represent percent of cell population in the following cell cycle phases: G1 (□), S (▒), and the sum of G2 + Mitotic (■). Error bars represent pooled standard deviation of three independent trials, each of which includes a minimum of 10^4 cell events analyzed. * indicates significant difference at a 95% confidence level, when compared to controls. Brackets indicate significant differences between increasing doses. **b** Flow cytometric analysis of GDM-1 population cell cycle distributions based on DNA content histograms, following 48 h of exposure to curdlan. Columns represent percent of cell population in the following cell cycle phases: G1 (□), S (▒), and the sum of G2 + Mitotic (■). Error bars represent pooled standard deviation of three independent trials, each of which includes a minimum of 10^4 cell events analyzed. *Significant difference at a 95% confidence level when compared to control. Brackets indicate significant differences between increasing doses

Discussion

An improved understanding of how airborne microorganisms and their components can lead to non-infectious adverse health effects is an important step toward the development of more targeted strategies in indoor air pollution mitigation and regulation. Damp spaces and water-damaged buildings have been associated with negative environmental health effects (Thrasher and Crawley 2009). Bioaerosols are widely regarded as a primary exposure hazard in this context; yet, despite numerous studies, the fundamental biological mechanisms underlying respiratory sensitivity to these environments

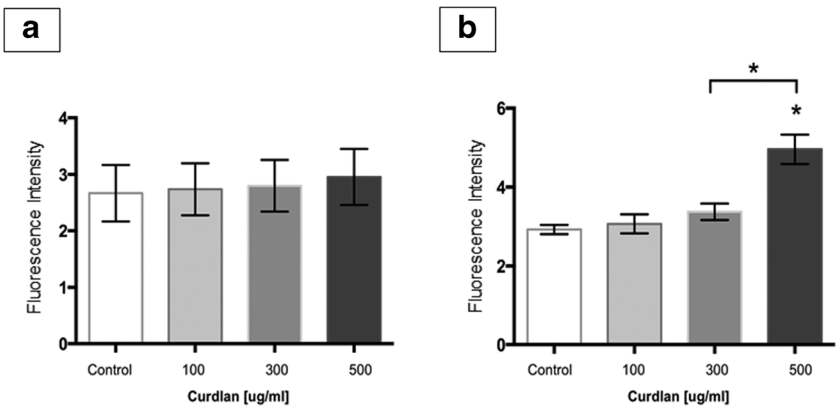

Fig. 3 **a** Intracellular ROS response of A549 epithelial cells exposed to increasing concentrations of curdlan as indicated by fluorescence intensity. Error bars represent pooled standard deviation of three independent trials, each of which includes a minimum of 10^4 cell events analyzed. No significant differences were detected. **b** Intracellular ROS response of GDM-1 epithelial cells exposed to increasing concentrations of curdlan as indicated by fluorescence intensity. Error bars represent pooled standard deviation of three independent trials, each of which includes a minimum of 10^4 cell events analyzed. *Significant differences at 95% confidence, when compared to control. Bracket indicates significant difference between increasing doses

remain unclear (Douwes et al. 2003). Comprehensive screenings of the non-allergenic effects caused by bioaerosol exposures, in ambient as well as indoor environments, are needed to identify bioaerosols of concern and to characterize the different modes of cellular stress that bioaerosol exposures can impart. Improved assessments can also inform the design of preventative measures aimed to reduce the risk of developing respiratory diseases caused and exacerbated by bioaerosols.

Bioaerosols have been shown to lead to the expression of inflammatory markers in cells exposed (Douwes et al. 2000); however, few studies have concurrently obtained converging lines of independent evidence to screen for dominant toxicological modes in a common cytometric platform (e.g., cyto, geno, and oxidative toxicity). Nor have such results been organized as a cohort matrix specifically designed to compare functionally disparate cellular components of the respiratory system.

Cytotoxicity challenges (Annexin V assays) were designed to reveal differences in both the magnitude and mode of cell death in response to curdlan exposure. This response varied significantly depending on the cell type challenged. While epithelial A549 cells displayed dose-dependent increases in cytotoxicity, phagocytic GDM-1 cells exposed to the same curdlan doses showed no significant cytotoxic response. Additionally, cell death in A549 cells was observed to occur via necrotic pathways, suggesting that exposure to curdlan can lead to physical and/or chemical damage to the cell membrane. This finding highlights the variation in sensitivity between the cell lines and supports the use of multiple cell lines and multiple reporting modes in future in vitro bioaerosol research.

Analyses were also conducted to evaluate the potential of curdlan to effect the cycle of DNA replication and cell division in populations (Sclafani and Holzen 2007). Cell cycle analyses from these challenges revealed unambiguous cell cycle alterations in both cell lines. As with the cytotoxicity experiments, cell cycle analyses indicated that epithelial A549 cells were more vulnerable to effects of curdlan than GDM-1 cells. Changes in DNA profiles of A549 cells suggest that exposure to higher levels of curdlan led to increasing numbers of cells which failed to complete replication of their DNA (cells halted in the "S" phase), as well as cells which replicated DNA but failed to successfully divide (cells halted in the G2/M phase). Unlike their epithelial counterparts, alterations in the DNA profiles of phagocytic GDM-1 cells manifest in an accumulation of cells halted predominantly in the S phase (cells failed to replicate DNA). Differences between the two cell lines illustrate that exposure to curdlan has the potential to induce markedly different types and magnitudes of cell cycle disturbances in functionally distinct respiratory cell lines. While cell cycle alteration is often related to DNA damage, progression through the phases of the cell cycle can also be delayed or blocked by a variety of structural defects in cells. Curdlan has been previously shown to enhance cell cycle arrest in tumor cells when used in combination with anti-cancer pharmaceutical drugs (Badulescu et al. 2009). While our research was undertaken for a different purpose, we report cell cycle results which are consistent with these cancer-related findings. Future research is needed to reveal the explicit mechanisms by which curdlan disrupts the cell cycle (e.g., quantification of protein kinases or s15p53) (Andrysik et al. 2011; Longhin et al. 2013).

Reactive oxygen species (ROS) are commonly present at low background concentrations in all healthy cells. However, bioaerosols and other pollutants can be potent inducers of oxidative stress, leading to the intercellular accumulation of ROS well above normal levels (Ball et al. 2000). Oxidative stress has the potential to lead various forms of cellular damage as a result of oxygen species' probability of reacting with other cellular biopolymers including proteins, lipids, and DNA (Li et al. 2002). Oxidative stress has also been related to respiratory system–wide pro-inflammatory effects and has been associated with asthma development (Li et al. 2003). Inhalation of PM is thought to cause oxidative stress via two general mechanisms. First, primary oxidant–generating properties of the compounds present in PM can induce redox cycling and the production of high levels of ROS within the cell (Delfino et al. 2011). Second, PM exposure can stimulate cell organelles to produce ROS (e.g., known as oxidative burst in macrophage cells) (Landreman et al. 2008). The two human cell lines used in this study were selected, in part, to observe the influence of both of these ROS-generating mechanisms. While GDM-1 cells are known to generate intracellular ROS during phagocytosis of particles, A549 epithelial cells do not engulf particles or produce an associated oxidative burst. Therefore, the ROS concentrations in A549 cells after exposure to curdlan provide a useful representation of only the primary oxidant–generating capacity of the curdlan (above normal background levels) and ROS concentrations observed in macrophage cells exposed to curdlan can provide information about the sum of both general types of ROS generation.

The accumulation of ROS in response to curdlan exposure has not been previously well characterized. We report here dose-dependent increases in intracellular ROS in GDM-1 cells, which did not occur in epithelial A549 cells. This finding suggests that observed increases of ROS in GDM-1 cells were predominately due to phagocytosis and aligns with published research which has shown that curdlan activates macrophage cells via molecular targets, referred to as pathogen-associated molecular patterns (PAMPs) (Kataoka et al. 2002). PAMPs are recognized

by the innate immune system and can stimulate immune cells to engulf particles of microbial origin (Goodridge et al. 2009). Internal ROS is generated by phagocytic cells during this process (oxidative burst) and likely accounts for observed increases in ROS in GDM-1 cells.

Intracellular accumulation of ROS, and the associated oxidative stress, is often implicated as a fundamental driver of PM toxicity because it has the potential to lead to DNA damage and cell death. Consequently, the quantification of oxidative stress and inflammatory biomarkers are among the most common analyses reported in bioaerosol and PM toxicity studies (Douwes et al. 2003; Fung and Hughson 2003). A549 cells were shown here to be more sensitive in both cytotoxicity and cell cycle assays than immature GDM-1 macrophage, yet A549 cells did not exhibit increases in ROS accumulation in response to curdlan exposure. These results indicate that the cytotoxicity and cell cycle alterations observed in A549 cells did not likely result from curdlan-induced oxidative stress. As intended in our suite design, this finding resulted from side-by-side analyses of multiple modes of toxicity and would have been undetected if only a singular biomarker or toxicological mode was assessed, as is common in the literature.

The enhanced resolution provided by this suite highlights that the use of a singular assay, or combination of assays focused on one type of cellular stress, may be insufficient for screening the toxicological effects of respiratory exposures. Further, these findings underscore that cell line selection for toxicological analyses of PM, including bioaerosols, can lead to differences in both the magnitude and mode of toxicological responses observed. Our results advocate the use of multiple cell lines and the examination of multiple modes of toxicity in in vitro studies. This work illustrates how multiple assays carried out in parallel on distinct cell lines can provide important insights into varying responses of the cellular components that make up the respiratory system. An example of the deductive power provided by this approach is the clear effect that curdlan exposure had on the cell cycle and cell death of A549 cells in the absence of ROS accumulation. While the assays are robust and informative, this suite was not designed to distinguish specific toxicological mechanisms. The aims of the suite-based analysis were to provide broad toxicological screening for PM/bioaerosol of interest, to identify general modes of toxicity, and to stimulate and provide focus for future research.

This research demonstrates how classic biochemical assays can be optimized across a common platform, for the purpose of creating an analytical matrix that can provide a more robust initial screening for PM toxicity research. While applied to curdlan in suspension, this approach can be extended to screen other classes of bioaerosols of immediate public health concern, as well as the study of the effects of mixtures of PM and a host of other airborne chemicals. The approach could be further improved upon by adapting the assays for use with an air-liquid interface.

References

Andrysik Z, Vondracek J, Marvanova S, Ciganek M, Neca J, Pencikova K, Mahadevan B, Topinka J, Baird WM, Kozubik A, Machala M (2011) Activation of the aryl hydrocarbon receptor is the major toxic mode of action of an organic extract of a reference urban dust particulate matter mixture: the role of polycyclic aromatic hydrocarbons. Mutat Res Fundam Mol Mech Mutagen 714(1–2):53–62. https://doi.org/10.1016/J.Mrfmmm.2011.06.011

Apetrei NS, Calugaru A, Badulescu MM, Lupu AR, Moscovici M, Mocanu G, Mihai D, Szegli G, Cremer L (2010) The effects of some Curdlan derivatives on Dectin-1 expression and cytokine production in human peripheral blood mononuclear cells. Roum Arch Microbiol Immunol 69(2):61–66

Badulescu MM, Apetrei NS, Lupu AR, Cremer L, Szegli G, Moscovici M, Mocanu G, Mihai D, Calugaru A (2009) Curdlan derivatives able to enhance cytostatic drugs activity on tumor cells. Roum Arch Microbiol Immunol 68(4):201–206

Ball JC, Straccia AM, Young WC, Aust AE (2000) The formation of reactive oxygen species catalyzed by neutral, aqueous extracts of NIST ambient particulate matter and diesel engine particles. J Air Waste Manage Assoc 50(11):1897–1903

Bardana EJ (2003) Indoor air quality and health: does fungal contamination play a significant role? Immunol Allergy Clin N Am 23(2):291–309

Beijer L, Thorn J, Rylander R (2002) Effects after inhalation of (1–>3)-beta-D-glucan and relation to mould exposure in the home. Mediat Inflamm 11(3):149–153. https://doi.org/10.1080/09622935020138181

Brown GD, Gordon S (2003) Fungal beta-glucans and mammalian immunity. Immunity 19(3):311–315

Carero D, Hoet P, Verschaeve L, Shoeters G, Nemery B (2001) Genotoxic effects of carbon black particles, diesel exhaust particles, and urban air particles and their extracts on a human alveolar epithelial cell line (A549) and human monocytic cell line (THP-1). Environ Mol Muanogen 37:155–163

Collins K, Jacks T, Pavletich N (1997) The cell cycle and cancer. Natl Acad Sci USA 94:2776–2778

Crook B, & Burton NC. (2010). Indoor moulds, sick building syndrome and building related illness. Fungal biology reviews, In Press, Corrected Proof

Delfino RJ, Staimer N, Vaziri NDJAQ, Atmos Health (2011) Air pollution and circulating biomarkers of oxidative stress. Air Qual Atmos Health 4(1):37–52. https://doi.org/10.1007/s11869-010-0095-2

Douwes J (2005) (1→3)-β-glucans and respiratory health: a review of the scientific evidence. Indoor Air 15:160–169

Douwes J, Wouters I, Dubbeld H, Zwieten LV, Steerenberg P, Doekes G, Heederik D (2000) Upper airway inflammation assessed by nasal lavage in compost workers: a relation with bioaerosol exposure. Am J Ind Med 37:459–468

Douwes J, Thorne P, Pearce N, Heederik D (2003) Bioaerosol health effects and exposure assessment: progress and prospects. Ann Occup Hyg 47(3):187–200. https://doi.org/10.1093/annhyg/meg032

Eisenbrand G, Pool-Zobel B, Baker V, Balls M, Blaauboer BJ, Boobis A, Carere A, Kleiner J (2002) Methods of in vitro toxicology. Food Chem Toxicol 40:193–236

Fabian MP, Miller SL, Reponen T, Hernandez MT (2004) Ambient bioaerosol indices for indoor air quality assessments of flood reclamation. J Aerosol Sci 36(5–6):763–783

Fung F, Hughson WG (2003) Health effects of indoor fungal bioaerosol exposure. Appl Occup Environ Hyg 18(7):535–544. https://doi.org/10.1080/10473220301451

Goodridge HS, Wolf AJ, Underhill DM (2009) Beta-glucan recognition by the innate immune system. Immunol Rev 230:38–50

Jin LH, Choi JK, Cho HS, Shim J, Kim YJ (2008) Microarray analysis of the gene expression profiles of SL2 cells stimulated by LPS/PGN and curdlan. Mol Cell 25(4):553–558

Kankkunen P, Teirila L, Rintahaka J, Alenius H, Wolff H, Matikainen S (2010) (1,3)-beta-glucans activate both dectin-1 and NLRP3 inflammasome in human macrophages. J Immunol 184(11):6335–6342. https://doi.org/10.4049/jimmunol.0903019

Kataoka K, Muta T, Yamazaki S, Takeshige K (2002) Activation of macrophages by linear (1right-arrow3)-beta-D-glucans. Impliations for the recognition of fungi by innate immunity. J Biol Chem 277(39):36825–36831. https://doi.org/10.1074/jbc.M206756200

Krishan A (1975) Rapid flow cytofluorometric analysis of mammalian cell cycle by propidium iodide staining. J Cell Biol 66(1):188–193

Kubala L, Ruzickova J, Nickova K, Sandula J, Ciz M, Lojek A (2003) The effect of (1–>3)-beta-D-glucans, carboxymethylglucan and schizophyllan on human leukocytes in vitro. Carbohydr Res 338(24):2835–2840

Landreman AP, Shafer MM, Hemming JC, Hannigan MP, Schauer JJ (2008) A macrophage-based method for the assessment of the reactive oxygen species (ROS) activity of atmospheric particulate matter (PM) and application to routine (daily-24 h) aerosol monitoring studies. Aerosol Sci Technol 42(11):946–957. https://doi.org/10.1080/02786820802363819

Lawniczek-Walczyk A, Gorny RL (2010) Endotoxins and beta-glucans as markers of microbiological contamination–characteristics, detection, and environmental exposure. Ann Agric Environ Med 17(2):193–208

Li N, Wang M, Oberley TD, Sempf JM, Nel AE (2002) Comparison of the pro-oxidative and proinflammatory effects of organic diesel exhaust particle chemicals in bronchial epithelial cells and macrophages. J Immunol 169(8):4531–4541

Li N, Hao M, Phalen RF, Hinds WC, Nel AE (2003) Particulate air pollutants and asthma: a paradigm for the role of oxidative stress in PM-induced adverse health effects. Clin Immunol 109(3):250–265. https://doi.org/10.1016/j.clim.2003.08.006

Longhin E, Holme JA, Gutzkow KB, Arlt VM, Kucab JE, Camatini M, Gualtieri M (2013) Cell cycle alterations induced by urban PM2.5 in bronchial epithelial cells: characterization of the process and possible mechanisms involved. Part Fibre Toxicol 10:63. https://doi.org/10.1186/1743-8977-10-63

Madureira J, Aguiar L, Pereira C, Mendes A, Querido MM, Neves P, Teixeira JPJAQ, Atmosphere, & Health (2018) Indoor exposure to bioaerosol particles: levels and implications for inhalation dose rates in schoolchildren. Air Qual Atmos Health 11(8):955–964. https://doi.org/10.1007/s11869-018-0599-8

McIntosh M, Stone BA, Stanisich VA (2005) Curdlan and other bacterial (1–>3)-beta-D-glucans. Appl Microbiol Biotechnol 68(2):163–173. https://doi.org/10.1007/s00253-005-1959-5

Nunez R. (2001). DNA measurement and cell cycle analysis by flow cytometry. In. Curr Iss Mol Biol (Vol. 3, pp. 67–70)

Rylander R (1994) Organic dusts and lung disease. Ann Agric Environ Med 1:7–10

Rylander R (2010) Organic dust induced pulmonary disease - the role of mould derived beta-glucan. Ann Agric Environ Med 17(1):9–13

Samadi S, Wouters IM, Houben R, Jamshidifard AR, Van Eerdenburg F, Heederik DJ (2009) Exposure to inhalable dust, endotoxins, beta(1->3)-glucans, and airborne microorganisms in horse stables. Ann Occup Hyg 53(6):595–603. https://doi.org/10.1093/annhyg/mep040

Schwarze PE, Totlandsdal AI, Lag M, Refsnes M, Holme JA, Ovrevik J (2013) Inflammation-related effects of diesel engine exhaust particles: studies on lung cells in vitro. Biomed Res Int 2013:685142–685113. https://doi.org/10.1155/2013/685142

Sclafani RA, Holzen TM (2007) Cell cycle regulation of DNA replication. Annu Rev Genet 41:237–280. https://doi.org/10.1146/annurev.genet.41.110306.130308

Thrasher JD, Crawley S (2009) The biocontaminants and complexity of damp indoor spaces: more than what meets the eyes. Toxicol Ind Health 25(9–10):583–615. https://doi.org/10.1177/0748233709348386

Turner J, Hernandez MT, Snawder J, McCabe KM (2014) An optimized analytical suite for comparing toxicity effects of diesel exhaust particles and their extracts on model human lung cells Aerosol Sci Tech Under Review

Vermes I, Haanen C, Steffens-Nakken H, Reutelingsperger C (1995) A novel assay for apoptosis. Flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled Annexin V. In J Immunol Methods 184:39–51 Netherlands

Williams DL (1997) Overview of (1–>3)-beta-D-glucan immunobiology. Mediat Inflamm 6(4):247–250. https://doi.org/10.1080/09629359791550