Endotoxin in concentrated coarse and fine ambient particles induces acute systemic inflammation in controlled human exposures

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ABSTRACT

Background Knowledge of the inhalable particulate matter components responsible for health effects is important for developing targeted regulation.

Objectives In a double-blind randomised cross-over trial of controlled human exposures to concentrated ambient particles (CAPs) and their endotoxin and (1→3)-β-D-glucan components, we evaluated acute inflammatory responses.

Methods 35 healthy adults were exposed to five 130-min exposures at rest: (1) fine CAPs (∼250 µg/m³); (2) coarse CAPs (∼200 µg/m³); (3) second coarse CAPs (∼200 µg/m³); (4) filtered air; and (5) medical air. Induced sputum cell counts were measured at screening and 24 h postexposure. Venous blood total leucocytes, neutrophils, interleukin-6 and high-sensitivity C reactive protein (CRP) were measured pre-exposure, 3 and 24 h postexposure.

Results Relative to filtered air, an increase in blood leucocytes 24 h (but not 3 h) postexposure was significantly associated with coarse (estimate=0.44×10⁹ cells/L (95% CI 0.01 to 0.88); n=132) and fine CAPs (0.68×10⁹ cells /L (95% CI 0.19 to 1.17); n=132), but not medical air. Similar associations were found with neutrophil responses. An interquartile increase in endotoxin (5.4 ng/m³) was significantly associated with increased blood leucocytes 3 h postexposure (0.27×10⁹ cells/L (95% CI 0.03 to 0.51); n=98) and 24 h postexposure (0.37×10⁹ cells/L (95% CI 0.12 to 0.63); n=98). This endotoxin effect did not differ by particle size. There were no associations with glucan concentrations or interleukin-6, CRP or sputum responses.

Conclusions In healthy adults, controlled coarse and fine ambient particle exposures independently induced acute systemic inflammatory responses. Endotoxin contributes to the inflammatory role of particle air pollution.

INTRODUCTION

It is well recognised that exposures to inhalable ambient particles are associated with significant morbidity and mortality.1–5 Knowledge of the particulate matter (PM) components responsible for the health effects observed in epidemiological studies is of importance for the development of targeted air pollution regulations.

While fine (PM₂.₅) particles are being widely regulated, there is more uncertainty over the toxicity of coarse (PM₂.₅–₁₀) particles.6 Fine particles, derived mainly from mobile and industrial emission sources, are associated with cardiovascular outcomes and are able to reach the alveolar region and deposit mainly by interception.7 Coarse particles have been shown to be associated with respiratory inflammation and disease as well as innate immune responses of airway macrophages,8 and tend to deposit by inertial impaction in the extrathoracic airways (nose and pharynx; above the vocal cords).9 Smaller coarse particles may also deposit onto the lower ciliated thoracic airways by gravitational sedimentation.10

Gram-negative bacterial cell walls contain endotoxin (lipopolysaccharide), which is composed of polysaccharide chains, a connecting core and a lipid A unit that is responsible for its toxic effects.10 Another ambient biological exposure that is highly correlated with endotoxin levels is (1→3)-β-D-glucan (hereafter glucan). These compounds are glucose polymers that are non-allergenic water-insoluble structural cell wall components of most fungi, as well as some bacteria and plants. Their biological activity is independent of cell viability and may be potentiated by the degree of chemical branching and intramolecular association (ie, single/triple helix or randomly coil structures).11

Human controlled exposure studies12–15 provide a unique opportunity to simulate air pollution levels like those seen regularly in cities like Beijing, China,16 while allowing for experimental control of the level of exposure. We used a double-blind randomised cross-over trial of controlled human exposures to coarse and fine concentrated ambient particles (CAPs) to evaluate their effects on acute pulmonary and secondary systemic inflammatory responses. In addition, we assessed whether exposure to increased concentrations of CAPs-associated endotoxin and glucan explained the inflammatory response to CAPs exposure.

METHODS

Study participants

We included 35 healthy non-smokers, aged 18–60 years, with no history of cardiovascular disease, hypertension (blood pressure >140/90 mm Hg) or diabetes. Subjects were not receiving...
treatment with cholesterol lowering medication or corticosteroids, and were free of respiratory tract infections for at least 3 weeks prior to exposure testing. Subjects were recruited from the University of Toronto Campus and surrounding area. The study was approved by the human research ethics committees of St. Michael’s Hospital, the University of Toronto and Health Canada. All participants provided written informed consent before enrolling.

Study design and exposure assessment
In a double-blind randomised cross-over block design, participants were exposed to five exposures: (1) fine CAPs between 0.1 and 2.5 microns aerodynamic diameter (∼250 µg/m³); (2) coarse CAPs between 2.5 and 10 microns aerodynamic diameter (∼200 µg/m³); (3) second coarse CAPs (∼200 µg/m³); (4) filtered air; and (5) medical air. Previous studies by our group had assessed the acute effects of fine CAPs.17–18 When we began this study, the effects of coarse CAPs were not well assessed. We added the second coarse exposure per subject to increase the power to assess effects on outcomes. The two coarse exposures were handled as separate treatments in the statistical analyses to increase variability and power. Each exposure lasted 130 min (120-min exposure plus an additional 10 min to complete all test measures), followed by a minimum 2-week washout period before the next exposure. Controlled exposures were generated using high-flow (5000 L/min) Harvard ambient particle concentrators.19–21 These particle concentration systems were used to draw ambient particles from a 1.8 m high PM10 inlet located, 10 m from a busy 4-lane downtown Toronto street with ~2500 vehicles passing during the 130-min exposure. Thus, traffic emissions were a major contributor to the ambient PM levels at this site. Ambient particle exposures were concentrated and adjusted through a dilution control system to deliver target concentrations of ~200 µg/m³ coarse CAPs and ~250 µg/m³ fine CAPs. The CAPs air stream was delivered directly to the subject seated inside a 4.9 m³ Lexan and steel tube frame enclosure, at rest and breathing freely (no mouthpiece) via an ‘oxygen type’ face-mask covering his/her nose and mouth. The study design called for rest so there would be no interference with the cardiovascular measure of flow-mediated dilation (not included in this manuscript). The delivery system was designed so that there were no visual cues as to the exposure type while participants were seated in the chamber.

For filtered air exposures, the coarse concentrator was run with a high-efficiency particulate air (HEPA) filter placed inline to remove the particles. For medical air exposures, compressed breathing-grade medical air was humidified to 30% relative humidity, passed through an inline HEPA filter and delivered to the subject at a flow rate of 30 L/min. Medical air was selected because it is free of gaseous and particulate pollutants and odours.

The original study design (randomised block) only included the first four exposures (no medical air). However, interim analysis showed greater than expected physiological responses with filtered air, which was designed to serve as the control exposure and thus was expected to induce a negligible effect. We hypothesised that these responses may have been due to ambient gases such as volatile organic compounds that can pass through the HEPA filter. Thus, a fifth exposure using medical air was added as a second additional control, although in the first 11 subjects it was always delivered to the subject as the last exposure in the series (ie, not randomised), but then randomised in later subjects. This deviation from the randomised block design was considered in statistical analyses.

Endotoxin and glucan were both collected on polycarbonate membrane filters during coarse and fine CAPs and filtered air (but not medical air) exposures. Filters were placed in pyrogen-free 15 mL French square bottles containing 5 mL of Limulus Amebocyte Lysate reagent water. After shaking for 20 min and mixing for 1 min with a vortex, samples were divided into two aliquots of 2.5 mL. To measure endotoxin, the sample was sonicated for 30 min at 26°C and then vortexed for another 1 min. The extract was analysed for endotoxin using Pyrochrome and Glucashield reagents following manufacturer’s instructions (Associates of Cape Cod, Inc. (ACC), East Falmouth, Massachusetts, USA). To measure glucan, 0.3 N NaOH was added to the sample, shaken on ice for 25 min and diluted with 10 mM NaOH. The extract was analysed for the amount of glucan using Glucatell reagent (ACC).

Outcome measures
We collected induced sputum at the screening visit (median 21 days prior to the first exposure treatment) and at 24 h postexposure. In this study, we used sputum total cell and neutrophil counts as outcomes. Venous blood was also collected from all study participants ~45 min prior to, and 3 and 24 h after the start of each exposure. We evaluated total blood leucocyte and neutrophil counts as well as blood interleukin-6 (IL-6) and high-sensitivity C reactive protein (hs-CRP) as markers of inflammation.

Statistical methods
We first described the baseline characteristics of the study participants and the exposure concentrations (mass, endotoxin and glucan) across the different treatments (coarse and fine CAPs and filtered/medical air). To account for the within-subject correlation in the outcome measures, while adjusting for daily physiological variability within subjects, we created new variables representing change. Therefore, blood outcomes were converted to: (1) 3 h post—pre change and (2) 24 h post—pre change. Sputum measures were converted to the 24 h post screening visit change. However, since the screening visit was a median 21 days prior to the first exposure treatment, we also performed sensitivity analyses using 24 h post-sputum measures as a single measure. We assessed the outcome measure distributions for normality, and if skewed, we transformed the data, as appropriate, prior to further analyses.

We used linear mixed effects models to account for the within-subject correlation in responses between the exposure treatments. We first examined whether, relative to filtered air, coarse and fine CAPs and medical air exposures were independently associated with each outcome. We included the four exposure types (treatments) as a categorical variable, and used filtered air as the control, which was randomised for all subjects by design. We then examined whether, accounting for the exposure type, variations in bioaerosol (endotoxin, and glucan) concentrations were associated with the inflammatory outcomes. Due to the collinearity in bioaerosol concentrations, we assessed the effect of endotoxin and glucan in separate models. Coarse and fine CAPs-associated bioaerosols were initially grouped together. We subsequently used interaction terms between bioaerosol concentrations and CAPs size fraction to examine whether associations between bioaerosol exposures and outcomes were modified by CAPs size fraction.

All models were tested as follows: (1) unadjusted, assuming the randomised design efficiently accounted for measured and unmeasured confounders; (2) adjusting for exposure order (1st–5th, as in table 3), to account for any potential stress or cumulative responses; and (3) adjusting for subjects’ age,
We did not measure bioaerosol concentrations in medical air.

We did not observe significant differences in subject characteristics (age, gender, ethnicity or BMI) between those with increased or decreased responses (results not shown).

Table 1 presents the associations between the controlled exposure treatments and the change in total blood leucocyte responses. While there appeared to be a response to filtered air (the reference group) 3 h postexposure in both the unadjusted model (intercept estimate = 0.50 \times 10^9 \text{ cells/L} (95\% \text{ CI 0.12 to 0.87})) and in model 1 adjusting for exposure order (estimate = 0.62 \times 10^9 \text{ cells/L} (95\% \text{ CI 0.20 to 1.05})), this was no longer statistically significant when we adjusted for potential confounders in model 2 (estimate = 0.60 \times 10^9 \text{ cells/L} (95\% \text{ CI 0.06 to 1.26})). Relative to filtered air, coarse and fine CAPs exposures were associated (estimate = 0.68 \times 10^9 \text{ cells/L (95\% CI 0.19 to 1.17)}), but not medical air (estimate = 0.36 \times 10^9 \text{ cells/L (95\% CI −0.20 to 0.93)}). We performed sensitivity analyses by removing marked responses, and found consistent results in models with blood neutrophil responses (see online supplementary material table 1).

Adjusting for treatment type (table 3, model 4), an interquartile increase in endotoxin (5.4 \text{ ng/m}^3) was significantly associated (estimate = 0.38 \times 10^9 \text{ cells/L (95\% CI 0.09 to 0.68)}) with an increase in blood leucocytes 3 h postexposure. While an interquartile increase in endotoxin concentration was associated (estimate = 0.37 \times 10^9 \text{ cells/L (95\% CI 0.12 to 0.63)}) with higher leucocytes 24 h postexposure (table 4, model 3), this association was no longer significant when we adjusted for treatment type (table 4, model 4). In a model excluding medical air exposures to obtain the same number of observations as in model 4 (results not shown in tables), coarse (estimate = 0.52 \times 10^9 \text{ cells/L (95\% CI 0.04 to 1.00)}) and fine CAPs (estimate = 0.74 \times 10^9 \text{ cells/L (95\% CI 0.21 to 1.27)}) remained significantly associated with higher leucocyte levels 24 h postexposure. However, when including endotoxin in model 4, the associations of coarse and fine CAPs became non-significant and the respective effect estimates were reduced by 40\% (0.52 to 0.31 \times 10^9 \text{ cells/L}) and 35\% (0.74 to 0.48 \times 10^9 \text{ cells/L}). The association between increases in endotoxin concentration and leucocyte responses 3 h (p for interaction = 0.67) or 24 h (p for interaction = 0.42) postexposure did not vary significantly by CAPs size fraction.

Variations in glucan concentrations were not associated with 3 or 24 h postleucocyte responses. Relative to filtered air, fine CAPs exposures were associated (estimate = −0.62 \mu\text{g/mL} (95\% CI −1.04 to −0.20), n = 132) with lower hs-CRP responses 24 h post-treatment (see online supplementary material table 2). This negative association did not remain significant (estimate = −0.23 \mu\text{g/mL} (95\% CI −0.62 to 0.15), n = 128) after we removed four marked responses presented (−2.6, 1.4, 2.2 and 3.7 \mu\text{g/mL}). We did not find any associations with blood IL-6 (see online supplementary material table 3) or sputum responses (see online supplementary material table 4).

**DISCUSSION**

In a double-blind randomised cross-over trial in 35 healthy adult subjects, coarse and fine ambient particle exposures were independently associated with an acute inflammatory response. The endotoxin content partially explained the inflammatory role of ambient particle exposures, and the effect did not differ between coarse and fine particles.

While we observed significant associations with systemic inflammatory responses (blood neutrophils), the lack of significant corollary findings in sputum may be due to insufficient...
power because of the small sample size. Nevertheless, human exposure studies of CAPs have generally not shown consistent results with induced sputum, with suggestions that systemic inflammation may be more pronounced than pulmonary responses.22–25 CAPs represent multiple sources of ambient pollutants rather than just diesel exhaust emissions. While we controlled total mass levels in our study, we were limited by not accounting for particle composition which varies with time due to changes in source emissions and prevailing environmental conditions. CAP composition also varies by study location, and therefore our results from Toronto may not represent what might be found elsewhere.25

Ambient PM constituents may include chemicals such as metals, organics and biological materials from bacteria, viruses and fungi. An in vitro study of rat alveolar macrophage (AM) cells found that endotoxin in urban air particles, but not in diesel particles, was responsible for inducing inflammatory cytokine expression.26 Humans may be more sensitive than animals to the effects of CAPs.22 Becker et al (2005)27 exposed AM cells from healthy adult subjects aged 20–35 years to fine and coarse particles in vitro and found that the main pro-inflammatory response was driven by the coarse size fraction, where the majority (~90%) of the stimulatory material in inhalable PM is known to be found.28 This stimulatory material is mainly derived from biological sources, and includes microbes and allergens.

Alexis et al (2006)8 exposed nine healthy subjects, on three separate occasions, to inhaled saline (0.9%, control), coarse PM collected from local ambient air in Chapel Hill, North Carolina, USA that was heated (20 h at 120°C) to inactivate biological material, or non-heated PM. Relative to saline, coarse PM exposure was associated with an increase in inflammatory polymorphonuclear leucocytes and macrophage mRNA tumour necrosis factor (TNF)-α, an upregulation of immune surface phenotypes on macrophages (mCD14, CD11b, HLA-DR), and increased phagocytosis, 2–3 h post inhalation. Biological inactivation was associated with lower mRNA TNF-α, phagocytosis and cell surface marker responses. Analysis of ambient coarse PM from Chapel Hill showed that it contained 30% gram-negative bacteria, with the remainder mostly composed of gram-positive cocci and fungal spores (Penicillium, Cladosporium).

We measured slightly higher levels of endotoxin in fine than in coarse CAPs (table 1). This may be due to the fact that
behbod potential benefits of PM2.5 exposure to the lung environment, subjects were, by design, exposed to higher concentrations of fine (\sim 250 \, \text{ng/m}^3) than coarse (\sim 200 \, \text{ng/m}^3) CAPs. Nevertheless, a separate epigenetic analysis of our study found coarse CAPs exposure to be associated with lowered toll-like receptor (TLR)-4 methylation, which can recognise the endotoxin component of coarse CAPs and trigger macrophages to release various inflammatory cytokines.29

Concentrated ambient endotoxin concentrations, irrespective of CAPs size fraction, were over 17-fold greater (geometric mean \sim 34 \, \text{EU/m}^3) than levels normally found in outdoor and

| Table 3 | Associations among 130-min controlled human exposure treatments, bioaerosols and 3-h changes in blood total leucocytes | 3 h post—pre change in leucocytes (# \text{cells}\times10^{9}/L) |
|----------|-------------------------------------------------|-----------------|-----------------|-----------------|
| Treatment (categorical) | Unadjusted (n=132) | Model 1 (n=132) | Model 2 (n=132) |
| | Estimate | 95% CI | Estimate | 95% CI | Estimate | 95% CI |
| Coarse CAPs | – | – | 0.25 | (–0.72 to 0.23) | – | – | 0.25 | (–0.26 to 0.75) |
| Fine CAPs | – | – | 0.37 | (–0.91 to 0.17) | – | – | 0.27 | (–0.24 to 0.78) |
| Medical air | – | – | – | – | – | – | – | – |
| Filtered air | Filtered air | Ref | Ref | Ref | Ref | Ref |

| Treatment (categorical) | Model 3 (n=98) | Model 4 (n=98) | Model 5 (n=80) | Model 6 (n=80) |
| | Estimate | 95% CI | Estimate | 95% CI | Estimate | 95% CI | Estimate | 95% CI |
| Coarse CAPs | – | – | 0.27* | (0.03 to 0.51) | – | – | 0.10 | (–0.06 to 0.25) |
| Fine CAPs | – | – | 0.38* | (0.09 to 0.68) | – | – | 0.07 | (–0.10 to 0.24) |
| Medical air | – | – | – | – | – | – | – | – |
| Filtered air | Filtered air | Ref | Ref | Ref | Ref | Ref |

| Treatment (categorical) | Model 3 (n=98) | Model 4 (n=98) | Model 5 (n=80) | Model 6 (n=80) |
| | Estimate | 95% CI | Estimate | 95% CI | Estimate | 95% CI | Estimate | 95% CI |
| Coarse CAPs | – | – | 0.31 | (–0.23, 0.85) | – | – | 0.39 | (–0.20, 0.98) |
| Fine CAP | 0.67** | (0.16 to 1.17) | 0.71** | (0.19 to 1.22) | 0.68** | (0.19 to 1.17) |
| Medical air | 0.21 | (–0.33 to 0.74) | 0.25 | (–0.24 to 0.93) | 0.36 | (–0.20 to 0.93) |
| Filtered air | Filtered air | Ref | Ref | Ref | Ref |

| Treatment (categorical) | Model 3 (n=98) | Model 4 (n=98) | Model 5 (n=80) | Model 6 (n=80) |
| | Estimate | 95% CI | Estimate | 95% CI | Estimate | 95% CI | Estimate | 95% CI |
| Coarse CAPs | – | – | 0.37** | (0.12, 0.63) | 0.25 | (–0.05, 0.56) | – | – |
| Fine CAP | – | – | – | – | – | – | – | – |
| Medical air | – | – | – | – | – | – | – | – |
| Filtered air | Filtered air | Ref | Ref | Ref | Ref |

| Treatment (categorical) | Model 3 (n=98) | Model 4 (n=98) | Model 5 (n=80) | Model 6 (n=80) |
| | Estimate | 95% CI | Estimate | 95% CI | Estimate | 95% CI | Estimate | 95% CI |
| Coarse CAPs | – | – | 0.09 | (–0.06, 0.25) | 0.07 | (–0.08, 0.22) |
| Fine CAP | – | – | – | – | – | – | – | – |

Note: age and BMI are centred at the mean.

Table 4 Associations among 130-min controlled human exposure treatments, bioaerosols and 24-h changes in blood total leucocytes
The lack of significant growth or water damage;32 a villa with excessive cells and identify microbial conserved structures of pathogens such as the family of TLRs, are found on antigen presenting phase proteins (APP) by the liver. CRP is an important APP that proliferation of B-lymphocytes and the production of acute phase proteins.

A review of studies on the potential effects of glucan on airway inflammation showed mixed results.34 This lack of consistency may be due to a number of reasons, such as small sample sizes, different exposure assessment methods, or lack of control for potential confounders or coexposures such as endotoxin.35–39 Furthermore, health effects vary by route of exposure (eg, inhalation/oral) and type of glucan. The Glucatell reagent used to measure glucan concentrations is specific to (1→3)-β-D-glucan and, therefore, cross-reactivity with plant glucan that has 1→4 linkages (as found in barley) does not occur. However, factor G activation has a bias for higher molecular weight glucan and single-helix and randomly coiled conformers over triple-helix structures. The ELISA method has been shown to be specific for fungi, (1→6) side-branched and (1→3)-β-D-glucan, as well as high molecular weight glucan, and may therefore be a better method for determining exposure to glucan likely to have important health effects.

IL-6 is a cytokine that stimulates neutrophil production, the proliferation of B-lymphocytes and the production of acute phase proteins (APP) by the liver. CRP is an important APP that functions as a soluble pattern recognition receptor (PRR). PRRs, such as the family of TLRs, are found on antigen presenting cells and identify microbial conserved structures of pathogen-associated molecular patterns. The exposure duration (130 min) and length of follow-up (24 h) was sufficient to elicit changes in IL-6.20 The lack of significant associations with IL-6 or CRP in this study may therefore be due to a number of other reasons, including: (1) healthy subjects may not be representative of the population susceptible to the inflammatory effects of CAPs exposure; (2) other cytokines may have been released by macrophages, such as IL-1, IL-8, TNF-α and platelet-activating factor; and (3) there may indeed have been no effects to detect.

Our study was limited by the short exposure durations and follow-up periods, which may not be completely representative of the spatiotemporal variability in real-life exposures. Due to collinearity of endotoxin and glucan exposures, we were unable to assess any effect measure modification of the association between endotoxin and inflammation by glucan. Furthermore, the selection of healthy adults may limit generalisability of study findings to susceptible subpopulation. A limitation was that we did not evaluate differential deposition and we did not do nasal lavage (it would have been problematic to do both nasal lavage and sputum evaluation). It is possible that the weak associations of coarse particles with sputum leucocytes counts relate, in part, to deposition patterns. Our primary endpoints in the main study from which our analyses came from were cardiovascular. Finally, we were limited in that we did not have the exposures prior to the chamber exposure. Due to the randomisation, the role of daily life exposures in the few days prior to each treatment were not expected to have had a differential impact on the association between exposures of varying size and health outcomes.

Despite these limitations, our study included a carefully standardised environment with well characterised exposures and physical activity levels. Our randomised study design enables subjects to serve as their own controls, thereby controlling for measured and unmeasured confounders. Circadian rhythms, physical activity and stress must be taken into account when analysing cytokines in peripheral blood; circadian rhythms were controlled for by standardising the time of day when exposure treatments were performed; subjects were seated at rest during treatments; and stress was accounted for by adjusting for exposure order in statistical analyses. Study team members and subjects were both unaware of their exposure assignment in this double-blind study, thereby preventing the introduction of bias.

CONCLUSIONS

We have shown that short duration controlled human exposures to coarse and fine CAPs were independently associated with acute systemic inflammatory responses in healthy non-smoking adults. Endotoxin contributes to the inflammatory role of both coarse and fine particle air pollution.

What this paper adds

In healthy adults, controlled coarse as well as fine ambient particle exposures independently induced acute systemic inflammatory responses.

Endotoxin contributes to the inflammatory role of particle air pollution.

Knowledge of the particulate matter components responsible for the health effects observed in epidemiological studies is of importance for the development of targeted air pollution regulations.

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Contributors

BB is the primary author and performed all statistical analyses.

BU, MS, JAS and FS were involved in the study design, data collection and analysis as well as write-up. LL, RP, BC, JS, PK and DRG were involved in study design, data analysis and write-up.

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Competing interests

None.
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