

Adhesion of *Streptococcus pneumoniae* to human airway epithelial cells exposed to urban particulate matter

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Background: Epidemiologic studies report an association between pneumonia and urban particulate matter (PM) less than 10 microns (μm) in aerodynamic diameter (PM_{10}). *Streptococcus pneumoniae* is a common cause of bacterial pneumonia worldwide. To date, the mechanism whereby urban PM enhances vulnerability to *S pneumoniae* infection is unclear.

Adhesion of *S pneumoniae* to host cells is a prerequisite for infection. Host-expressed proteins, including the receptor for platelet-activating factor (PAFR), are co-opted by *S pneumoniae* to adhere to lower airway epithelial cells.

Objectives: To define whether inhalable urban PM enhances the adhesion of *S pneumoniae* to airway epithelial cells.

Methods: A549 cells were cultured with PM_{10} from Leicester (United Kingdom [UK]) and PM_{10} and PM less than $2.5 \mu\text{m}$ in aerodynamic diameter ($\text{PM}_{2.5}$) from Accra (Ghana), then infected with *S pneumoniae* strain D39. Pneumococcal adhesion to human primary bronchial epithelial cells was also assessed. Bacterial adhesion was determined by quantitative culture and confocal microscopy. The role of oxidative stress was assessed by N-acetyl cysteine, and the role of PAFR was assessed by mRNA transcript level, receptor expression, and receptor blocking. **Results:** PM_{10} (UK) increased *S pneumoniae* adhesion to both A549 airway epithelial cells and human primary bronchial epithelial cells. PM_{10} (Ghana) and $\text{PM}_{2.5}$ (Ghana) also increased adhesion. Culture of A549 cells by PM_{10} (UK) increased PAFR mRNA transcript level and PAFR expression. PM_{10} (UK)-stimulated adhesion to A549 cells was attenuated by a PAFR blocker and N-acetyl cysteine.

Conclusion: Urban PM increases adhesion of *S pneumoniae* to human airway epithelial cells. PM-stimulated adhesion is

mediated by oxidative stress and PAFR. (J Allergy Clin Immunol 2011;■■■:■■■-■■■.)

Key words: Urban particulate matter; *Streptococcus pneumoniae*, adhesion, A549, primary human bronchial epithelial cells

Pneumonia is responsible for 1 in 5 deaths in young children in low-income countries¹ and is a cause of acute respiratory morbidity in high-income countries.² Strains of *Streptococcus pneumoniae* (pneumococci) with the capacity to achieve firm adherence to epithelial cells and invade the airway epithelial barrier are responsible for the majority of cases of bacterial pneumonia.^{3,4} To adhere to airway cells, *S pneumoniae* co-opts host proteins. For example, *S pneumoniae* lipoteichoic acid has phosphorylcholine modifications that are similar to that of the natural ligand for platelet-activating factor.⁵ Thus, activation of platelet-activating factor receptor (PAFR) expression on airway epithelial cells leads to increased adhesion and invasion of *S pneumoniae*,⁶ blocking PAFR attenuates pneumococcal adhesion^{6,7} and PAFR^{-/-} mice are relatively resistant to pneumococcal pneumonia.⁸

Epidemiologic studies suggest that air pollution from cigarette smoke and vehicular emissions increase vulnerability to bacterial pneumonia.⁹⁻¹² The component of air pollution responsible for this association is unclear; however, animal and cell studies suggest a major role for carbonaceous particulate matter (PM) in mediating adverse health effects.^{13,14} Potentially inhalable PM is defined by mass (μg), volume of air (m^3), and size ($<10 \mu\text{m}$ or $<2.5 \mu\text{m}$ aerodynamic diameter; PM_{10} or $\text{PM}_{2.5}$).¹³ A common component of urban PM, whether from vehicular emissions typical of high-income countries or from a mix of vehicular and solid fuels typical of low-income countries, is aggregates of elemental carbon nanoparticles.^{15,16} Inhaled carbon nanoparticles have the capacity to be retained by lung tissue,¹⁵ induce pulmonary oxidative stress,¹⁷ and stimulate proinflammatory cytokine release from airway cells.¹⁸ Because *S pneumoniae* is the major cause of bacterial pneumonia, we hypothesized that urban PM, via oxidative stress, increases the vulnerability of lower airway cells to pneumococcal infection. We therefore sought to determine the effect of urban PM on the prerequisite step for infection; ie, pneumococcal adhesion to human airway cells.

METHODS

Cells and *S pneumoniae*

A549, a human type II pneumocyte cell line, was obtained from Sigma-Aldrich (Poole, Dorset, United Kingdom [UK]) and maintained in Dulbecco modified Eagle medium (Invitrogen Ltd, Paisley, UK) with 10% FBS and 1% L-glutamine/penicillin-streptomycin (Lonza Ltd, Basel, Switzerland). Human primary bronchial epithelial cells (HBEPc) were obtained from the main bronchi of a healthy white male and were purchased from Promocell

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Abbreviations used

CFU:	Colony-forming units
Ct:	Cycle time
GAPDH:	Glyceraldehyde-3-phosphate dehydrogenase
HBEpC:	Primary human bronchial epithelial cells
LDH:	Lactate dehydrogenase
MTT:	3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide
NAC:	N-acetyl cysteine
PAFR:	Platelet-activating factor receptor
PM:	Particulate matter
PM _{2.5} :	Particulate matter less than 2.5 microns in aerodynamic diameter
PM ₁₀ :	Particulate matter less than 10 microns in aerodynamic diameter
SEM:	Standard error of the mean
UK:	United Kingdom

(Heidelberg, Germany). The virulent type 2 *S pneumoniae* encapsulated strain D39 (NCTC 7466) was purchased from the National Collection of Type Cultures (Central Public Health Laboratory, London, UK) and grown to mid-logarithmic phase (OD 600 = 0.5) before it was added to airway epithelial cells at 200 μ L 2.4×10^9 CFU/mL. Because D39 is a slow-growing strain, infection dose was optimized at 4.8×10^8 per well (2400 bacteria per cell).

PM

PM₁₀ (UK) was collected on TX40 Teflon-coated glass fiber filter cartridges (Air Monitors Ltd, Tewkesbury, UK) by a fixed tapered element oscillating microbalance (Series 1400a TEOM Control Unit; Rupprecht and Patashnick Co, Inc, East Greenbush, NY) in the central area of Leicester, a medium-sized UK city where most ambient PM₁₀ is from fossil fuel combustion.¹⁹ Urban PM from Ghana was collected, as previously reported,¹⁷ from an area of Accra where air pollution is from a mix of road traffic, charcoal smoke, and wood smoke.²⁰ PM₁₀ and PM_{2.5} from Accra (Ghana) were collected on polytetrafluoroethylene filters with rings (Pall Life Sciences, Ann Arbor, Mich). Urban PM_{2.5} (Ghana) was sampled by using a ChemComb Model 3500 Speciation Sampling Cartridge (Thermo Fisher Scientific Inc, Waltham, Mass) with an upper size cutoff of 2.5 μ m, and urban PM₁₀ (Ghana) was sampled by a Harvard impactor with a cutoff of 10 μ m. PM was extracted from filters by sonication in PBS (Sigma-Aldrich, Gillingham, UK) for 5 minutes and the extracted dose (μ g/mL) indexed to the OD of known concentrations of ultrafine carbon black in PBS (Printex 90, Degussa, Germany, a gift from Professor Ken Donaldson, Edinburgh Medical School, UK) by using the optical absorbance method of Brown et al.²¹

Pneumococcal adhesion

Adhesion of pneumococci to airway cells was assessed by using a standard bacterial adhesion assay.^{6,22} Briefly, airway cells at 2×10^5 /mL were seeded in 24-well plates (Costar; Sigma-Aldrich). Urban PM was added to cell monolayers and incubated for 4 hours at 37°C. Nonadherent PM was removed by washing twice with Dulbecco modified Eagle medium and pneumococci added and incubated for 2 hours.⁷ Cell monolayers were washed 5 times to remove loosely attached pneumococci. Cells were removed from the tissue culture plate by trypsin-EDTA and lysed with ice-cold sterile distilled water for 10 minutes. Serial lysate dilutions were plated for quantitative bacterial culture and assessment of colony-forming unit (CFU) count per mL.²³ To assess the fraction of pneumococci that had penetrated into cells, adherent cell surface bacteria were first killed by gentamicin (200 μ g/mL) and penicillin G (10 μ g/mL). Intracellular bacteria that were protected from antibiotics were recovered by cell lysis using ice-cold sterile water.^{6,22} We established, as previously reported,²⁴ that urban PM₁₀ does not directly stimulate *S pneumoniae* proliferation. In addition, we established that extract of blank filter matrix does not stimulate adhesion. Pneumococcal adhesion was also assessed directly by

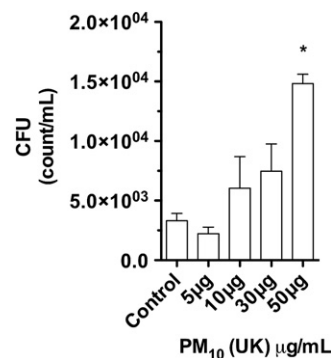


FIG 1. Effect of urban PM₁₀ (UK) on the adhesion of *S pneumoniae* D39 to A549 cells. PM₁₀ dose was indexed to ultrafine carbon black by using the optical absorbance of Brown et al.²¹ Cells were cultured with PM₁₀ for 4 hours, then infected with *S pneumoniae* for 2 hours. Adherence of bacteria was determined by quantitative culture and is expressed as CFU count/mL. Incubation with PM₁₀ (UK) at 50 μ g/mL increases CFU count (* $P < .001$ by *t* test). Data are represented as means and SEMs ($n \geq 5$) and are representative of >3 separate experiments.

confocal microscopy. Briefly, A549 cells were stained with Hoescht stain (10 μ g/mL, Invitrogen Ltd) for 45 minutes at 37°C, and *S pneumoniae* was stained with bis-oxonal dye (Bis - (1,3 - dibutylbarbituric acid) pentamethine oxonol, 10 μ g/mL; Sigma-Aldrich) for 10 minutes at room temperature. Fluorescent pneumococci were washed before adding to cell monolayers and adherent bacteria visualized by using a Zeiss LSM510 Meta confocal microscope (Carl Zeiss, Welwyn Garden City, UK) with a 63 $\times$ /1.4 NA Plan Apochromat objective and Zeiss software. Images were analyzed using the Cell Scoring plug-in in Metamorph (MDS Analytical Technologies, Wokingham, UK). Pneumococcal fluorescence was determined from 50 A549 cells from at least 5 randomly selected high-power fields. PM₁₀ and pneumococcal interactions with A549 cells were visualized by both transmission and scanning electron microscopy (see this article's Methods in the Online Repository at www.jacionline.org).

Cell viability

Cell viability was assessed by using lactate dehydrogenase (LDH) release according to the manufacturer's protocol (Sigma-Aldrich). LDH release was expressed as a percentage of release from total cell lysis. Cell cytotoxicity was assessed by the 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT) assay.²⁵ Briefly, 20 μ L MTT (5 mg/mL; Sigma-Aldrich) in PBS was added to cells, and incubated for 1 hour. Medium was removed and 100 μ L of dimethyl sulfoxide (Sigma-Aldrich) added for 30 minutes. Absorbance was read at 550 nm. Values are expressed as % MTT absorbance of medium control.

Oxidative stress

To assess the role of oxidative stress, A549 cells were incubated with N-acetyl cysteine (NAC; Sigma-Aldrich) 30 mmol/mL for 1 hour before incubation with *S pneumoniae*. The effect of NAC on intracellular glutathione was measured by reverse-phase HPLC with electrochemical detection (see this article's Methods in the Online Repository).

PAFR

Platelet-activating factor receptor transcript levels were assessed by quantitative RT-PCR. Briefly, 2×10^5 A549 cells were harvested for each sample, and RNA was prepared by using the RNeasy mini kit (Qiagen, Crawley, UK). Random hexamers (Qiagen) and Moloney murine leukemia virus reverse transcriptase (Invitrogen Ltd) were used to synthesize first-strand cDNA according to the manufacturer's instructions. mRNA transcript level of PAFR and the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were determined by quantitative real-time PCR using an

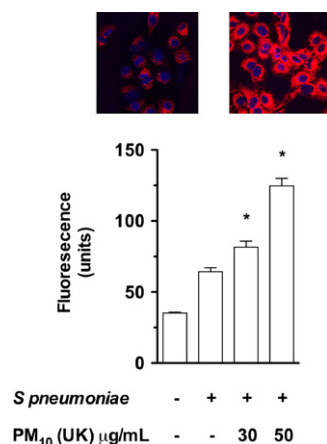


FIG 2. Adhesion of D39 *S pneumoniae* to A549 cells assessed by confocal microscopy. A549 cells were stained by Hoescht stain, then cultured with medium or PM₁₀ (UK) 50 µg/mL, then cultured with *S pneumoniae* (n = 5 for each data point). Culture of cells with PM₁₀ (UK) increases mean *S pneumoniae* fluorescence. Data are represented as means and SEMs (n = 50 cells) and are representative of 3 separate experiments. *P < .01 by t test. Left panel shows a representative image of A549 cells cultured in medium. Adherent *S pneumoniae* are red. Right panel shows cells cultured with 50 µg/mL PM₁₀ (UK) then cultured with *S pneumoniae*.

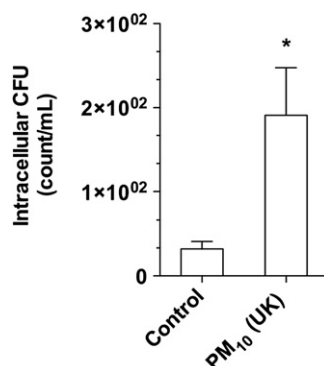


FIG 3. A549 cells cultured with PM₁₀ (UK) 50 µg/mL and treated with antibiotics to kill cell-surface *S pneumoniae* leaving only intracellular bacteria. CFU was determined by quantitative culture. Incubation of cells with PM₁₀ (UK) increases the intracellular CFU count. *P < .01 versus medium control by t test. Data are represented as means and SEMs (n ≥ 5) and are representative of >3 experiments.

ABI 7500 real-time PCR system (Applied Biosystems, Warrington, UK) with TaqMan primer and probe sets HS00265399_S1 (PAFR) and Hs99999905_m1 (GAPDH), respectively. mRNA transcript level was normalized to GAPDH and the relative change in expression between samples calculated by using the comparative cycle time (Ct) method according to the manufacturer's instructions (Applied Biosystems, Paisley, UK). PAFR expression was assessed by using confocal microscopy. Control cells and cells treated with PM₁₀ (UK) for 4 hours were fixed with 4% paraformaldehyde, blocked by using 10% BSA, and incubated with a dilution of 1/1000 mouse antihuman PAFR mAb (Cayman Chemical, Ann Arbor, Mich). Immunostaining of PAFR was achieved by incubation for 1 hour with Hoescht stain (10 µg/mL) and an Alexa 488-labeled antimouse secondary antibody at a dilution of 1/500 (Invitrogen Ltd). Cells were imaged by confocal microscopy as described. PAFR fluorescence was determined by an operator blind to treatment from at least 50 cells from 5 randomly selected high-power fields of each treatment, and expressed as mean fluorescence per cell. We established that neither the primary antibody alone nor the secondary antibody alone increased fluorescence. The functional importance of PAFR was assessed by co-incubation of PM₁₀

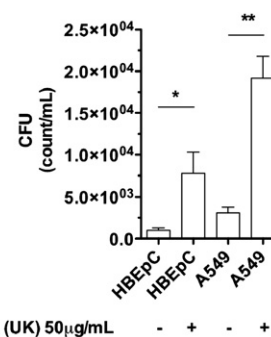


FIG 4. Adhesion of D39 *S pneumoniae* to human primary bronchial epithelial cells (HBEPC) cultured with 50 µg/mL PM₁₀ (UK). PM₁₀ increases pneumococcal adhesion in primary cells. Basal and stimulated adhesion in primary cells is lower than A549 cells. *P < .05, **P < .01 versus medium control by t test. Data are represented as means and SEMs (n ≥ 5) and are representative of >3 separate experiments.

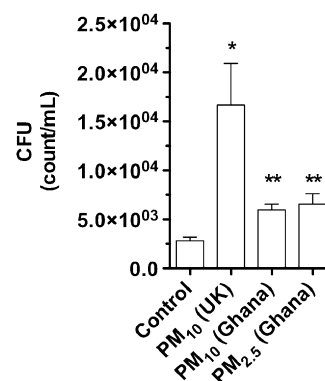


FIG 5. Effect of urban PM₁₀ and PM_{2.5} (Ghana) on the adhesion of *S pneumoniae* to A549 cells. Urban PM₁₀ and PM_{2.5} (Ghana) increases pneumococcal adhesion. *P < .05, **P < .01 versus medium control by t test. PM (Ghana)-stimulated adhesion is lower than PM₁₀ (UK)-stimulated adhesion (P < .05). Data are represented as means and SEMs (n ≥ 5) and are representative of 3 separate experiments.

(4 hours) with a PAFR blocker (10 µmol/mL WEB 2086; Tocris Bioscience, Avonmouth, UK)^{26,27} previously reported to attenuate cytokine-stimulated adhesion of *S pneumoniae* to A549 cells.⁶

Statistical analysis

Data are summarized as means (SEMs) and analyzed by using GraphPad Prism version 5.03 (GraphPad Software Inc, La Jolla, Calif). Analyses were performed by 1-way ANOVA, then unpaired t test. Results were validated by at least 3 separate experiments performed at different times. For mRNA transcript number, the mean of raw data of control samples (n = 5) was assigned 100% and the SEM converted to a percentage of the raw value. A P value < .05 was considered significant.

RESULTS

Culture of A549 cells with urban PM₁₀ (UK) increased adhesion of *S pneumoniae* assessed by quantitative culture (P < .001 vs medium control at 50 µg/mL; Fig 1). Confocal microscopy of fluorescent *S pneumoniae* confirmed that PM₁₀ (UK) stimulated a dose-dependent increase in pneumococcal adhesion to A549 cells (P < .01 vs medium control; Fig 2). Increased pneumococcal adhesion to A549 cells resulted in increased intracellular penetration of bacteria (P < .01; Fig 3). The same pattern of

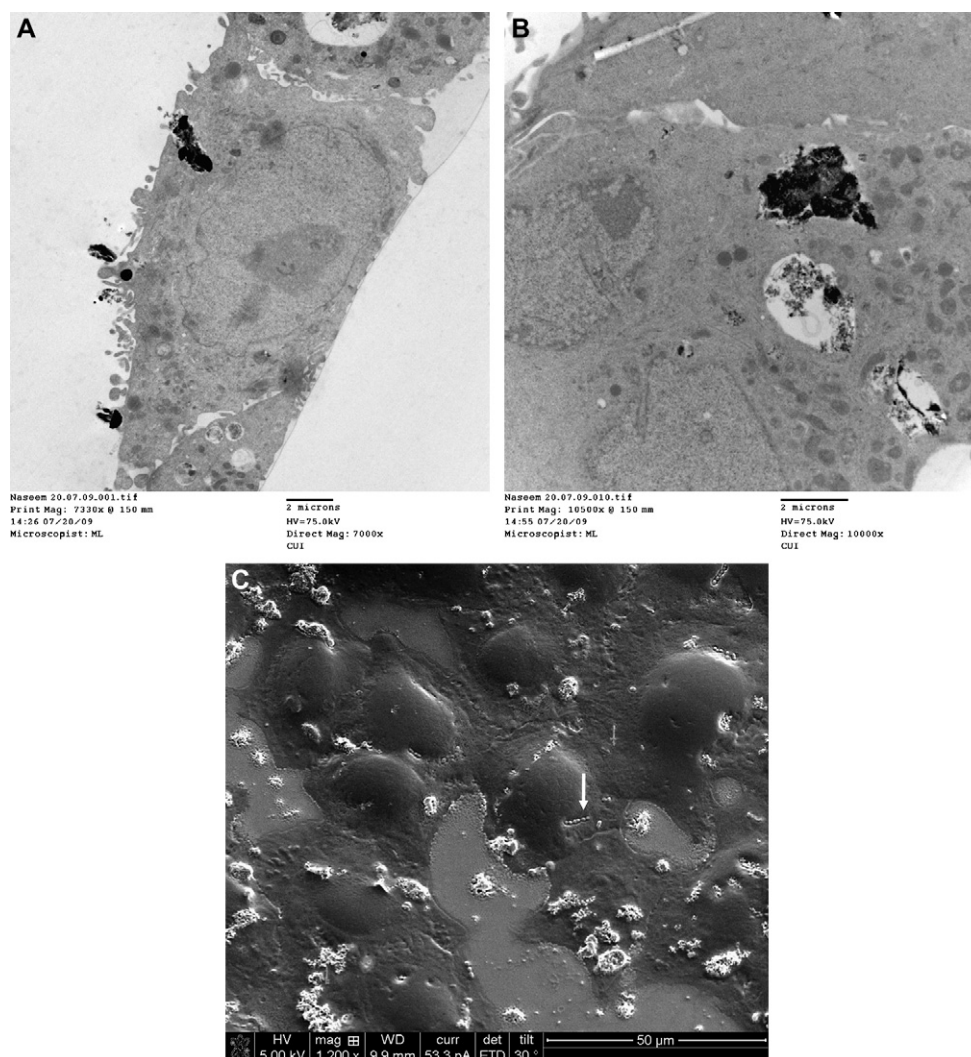


FIG 6. **A**, Transmission electron microscopy (TEM) image of an A549 cell cultured with PM₁₀ (UK). Black electron-dense material represents PM. Bar = 2 μm, ×7000. **B**, TEM of an A549 cell under higher magnification showing PM₁₀ (UK) both within intracellular vesicles and free in the cytoplasm. Bar = 2 μm, ×10,000. **C**, Scanning electron microscopy image of A549 cells after culture with PM₁₀ (UK) and *S. pneumoniae*. The white irregular surface material is PM, and adherent pneumococci are beadlike structures in cell-surface depressions (arrow). Bar = 50 μm, ×1200.

adhesion was observed in human primary bronchial epithelial cells cultured with PM₁₀ (UK; $P < .05$ vs medium control; Fig 4) and in A549 cells cultured with urban PM₁₀ and PM_{2.5} from Ghana ($P < .01$ vs control; Fig 5). However, stimulation of adhesion by PM (Ghana) was reduced compared with PM₁₀ (UK; Fig 5).

Electron microscopy showed PM₁₀ (UK) both firmly attached to the surface of A549 cells and penetrating into cells (Fig 6, A and B). Pneumococci were seen in cell surface depressions (Fig 6, C) with no direct binding of bacteria to PM₁₀. PM₁₀ (UK) at 50 μg/mL did not increase LDH release (see this article's Fig E1 in the Online Repository at www.jacionline.org) and did not reduce cell metabolic stability (MTT $99 \pm 11\%$ of control level; $P = \text{NS}$ vs control).

Treatment of A549 cells with NAC increased intracellular glutathione (mean glutathione [95% CI], 233 [187-278] vs 505 [430-579] μmol/mg protein), and NAC partly attenuated pneumococcal adhesion stimulated by PM₁₀ (UK; $P < .05$; Fig 7).

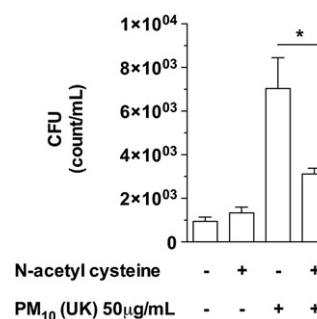


FIG 7. Effect of the antioxidant NAC (30 mmol/mL) on PM₁₀ (UK)-stimulated adhesion of *S. pneumoniae* to A549 cells. The number of adherent bacteria was determined by quantitative culture and expressed as CFU/mL. Incubation of A549 cells with N-acetyl cysteine (NAC) before infection partly attenuates the PM₁₀ (UK)-stimulated increase in CFU count. * $P < .05$ versus PM₁₀ without NAC by t test. NAC has no significant effect on CFU count in medium control cells ($P = \text{NS}$ versus cells without NAC). Data are shown as means and SEMs ($n = 5$) and are representative of 3 separate experiments.

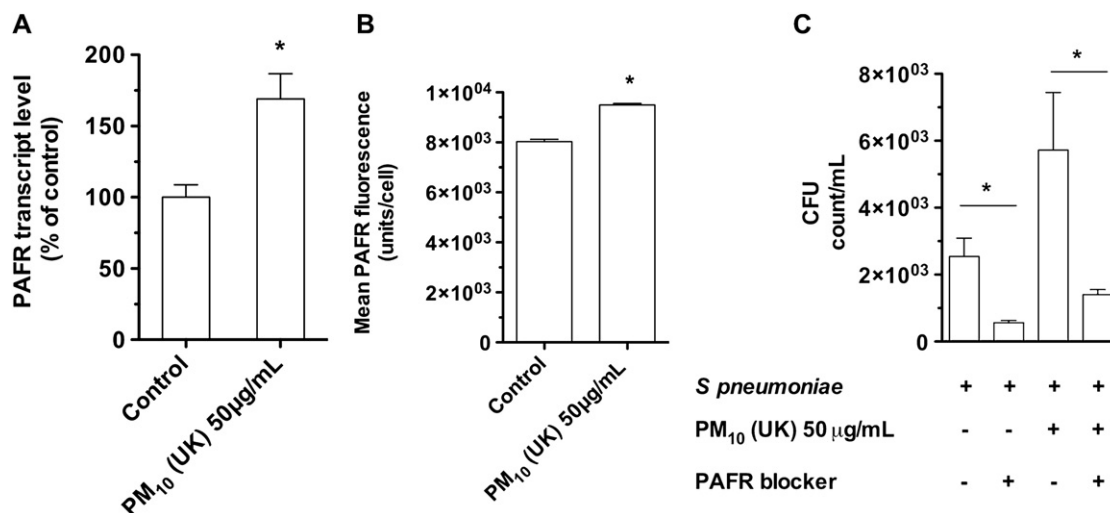


FIG 8. **A**, PAFR transcript level in A549 cells cultured with PM₁₀ (UK) 50 μg/mL for 4 hours. Transcript level was assessed by quantitative RT-PCR and normalized for the housekeeping gene GAPDH. The relative change in expression was calculated by using the comparative Ct method, and data expressed as % of control. Data show means and SEMs (n = 5 in triplicate wells). PM₁₀ (UK) increased PAFR transcript level. **P* < .01 versus control by *t* test. **B**, PAFR expression on A549 cells assessed by confocal microscopy. A549 cells were cultured with PM₁₀ (UK) 50 μg/mL for 4 hours. Mean fluorescence per cell was determined for 100 cells. PM₁₀ (UK) increases PAFR expression. Data are represented as means and SEMs (n = 50 cells) and are representative of 3 separate experiments. **P* < .001 by *t* test. **C**, Effect of PAFR blocker WEB2086; 10 μmol/mL on PM₁₀ (UK)-stimulated adhesion of *S pneumoniae* to A549 cells. The amount of adherent bacteria is expressed as CFU. Blocking PAFR attenuates both basal adhesion and the PM₁₀ (UK)-stimulated increase in CFU count. **P* < .05 versus PM₁₀ without PAFR blocker, by *t* test. Data are represented as means and SEMs (n = 5) and are representative of >3 separate experiments.

Culture of A549 cells with PM₁₀ (UK) increased PAFR transcript level (*P* < .01; Fig 8, A) and increased surface expression of PAFR (*P* < .001; Fig 8, B). PM₁₀ (Ghana) also increased PAFR surface expression (data not shown). Pneumococcal adhesion stimulated by PM₁₀ (UK) was attenuated by co-incubation with a PAFR blocker, WEB 2086 (Fig 8, C)

DISCUSSION

In this study, we found that inhalable PM from urban areas in both the developing world and the developed world increases the adhesion of pneumococci to human airway epithelial cells. Pneumococcal adhesion stimulated by urban PM is associated with enhanced vulnerability to cellular infection. We excluded the possibility that increased pneumococcal adhesion is due to cell death or injury by the absence of LDH release and MTT expression. Electron microscopy of PM-exposed cells showed PM attached to the A549 cell surface and in intracellular vesicular structures. Similarly, Calcabrini et al¹⁸ reported that electron microscopy of A549 cells exposed to urban PM_{2.5} showed “firm adhesion” of PM to the cell surface and PM in “vesicular structures” and free within the cytoplasm.

To date, the most convincing evidence that urban PM increases vulnerability to pneumococcal pneumonia is from epidemiologic studies. For example, Barnett et al¹⁰ reported a 2.4% (95% CI, 0.1–4.7) increase in hospital admissions for doctor-diagnosed “pneumonia or bronchitis” in young children for each interquartile increase in urban PM_{2.5}, and Neupane et al¹¹ reported that long-term exposure to urban PM_{2.5} is associated with hospitalization of adults with community-acquired pneumonia. Increased vulnerability to pneumonia has also been reported with exposure

to solid and biomass fuel emissions and tobacco smoke.^{28,29} It is unclear why Ghana PM (a mix of fossil fuel, solid fuel, and biomass fuel emissions) is less effective in stimulating adhesion compared with predominately fossil fuel PM from the UK. We speculate that combustion source is an important determinant of the capacity of PM to stimulate adhesion. Indeed, a recent exposure study in healthy volunteers reported that wood smoke is less toxic to the airway cells than diesel exhaust emissions.³⁰ Further adhesion studies are therefore required using PM from single sources such as diesel, wood, tobacco, and charcoal.

The ability of combustion PM to induce oxidative stress in airway cells is considered to be an important factor in the initiation of adverse health effects.³¹ We have previously reported that PM from biomass and fossil fuels depletes antioxidants *in vitro*.³² In the current study, increasing intracellular glutathione by NAC partly attenuates PM-stimulated adhesion. Similarly, NAC pretreatment of rats exposed to urban air PM attenuates pulmonary free radical formation and reduces lung edema.³³ The components of urban PM responsible for oxidative stress in the current study remain unclear. Potential sources of oxidative stress are carbon nanoparticles *per se* and metals adsorbed onto the carbon surface.^{32,34} It is unlikely that endotoxin contamination of PM is a major stimulus for adhesion because endotoxin-free ultrafine carbon black (14 nm) also increases pneumococcal adhesion to A549 cells (data not shown).

Platelet activating factor receptor is a putative receptor for PM-stimulated pneumococcal adhesion to airway cells. In A549 cells, increased pneumococcal adhesion is stimulated by IL-1 and is attenuated by a PAFR blocker.⁶ In human tracheal cells, Ishizuka et al⁷ reported that acid stimulates pneumococcal adhesion, and this is associated with upregulation of

PAFR mRNA and increased PAFR surface expression. Similarly, we found that PM₁₀ (UK) increased the PAFR transcript level and PAFR surface expression in A549 cells and that co-incubation with a PAFR blocker (WEB 2086) attenuated adhesion. These data suggest that PAFR is a host receptor mediating PM-stimulated pneumococcal adhesion to lower airway cells.

There are limitations to our study. First, exposure of epithelial cells to PM *in vivo* is via deposition at an air-tissue interface and not via deposition from an aqueous suspension. Second, it is unclear whether a PM dose of 50 µg/mL reflects exposure of airway cells *in vivo*. Delfino et al³⁵ reported personal PM exposure peaks of over 150 µg/m³ in urban children. In Accra (Ghana), we have previously reported PM₁₀ concentrations as high as 100 µg/m³.³⁶ In homes burning biomass fuels, peak carbonaceous PM exposure is much higher—reaching thousands of µg/m³.³⁷ Over the long term, biomass smoke exposure results in visible black staining of the bronchial surface.³⁸ Another factor that increases exposure of airway cells is turbulent air flow—which results in “hot spots” of greatly increased PM deposition.³⁹ Third, our model does not assess compensatory effects of other cells of the human airway innate immune defense system—for example, alveolar macrophages. However, PM effects have been reported in a fully functional innate immune system. For example, *S pneumoniae* infection is increased in mice exposed to 50 µg concentrated urban PM.⁴⁰ Finally, the A549 cell line may not fully reflect responses of lower airway cells *in vivo*. Increased adhesion is likely to be common feature of lower airway epithelial cells because we also found increased adhesion in human primary bronchial epithelial cells. Compatible with a previous study,⁴¹ pneumococcal adhesion to primary cells tended to be lower than A549 cells.

In conclusion, urban inhalable PM from both the UK and Ghana stimulates pneumococcal adhesion to human lower airway cells. Increased pneumococcal adhesion is associated with increased invasion of cells by bacteria. Oxidative stress mediates the adhesive response of airway epithelial cells, and PAFR is a receptor co-opted by pneumococci to facilitate PM-stimulated adhesion.

We thank Mr Simon Pollard (Leicester City Council) for supplying the PM₁₀ (UK) filters.

Key messages

- Urban particulate matter increases the adhesion and invasion of *S pneumoniae* in human lower airway cells *in vitro*.
- Stimulation of pneumococcal adhesion by urban PM is mediated partly via oxidative stress.
- PAFR is co-opted by *S pneumoniae* to adhere to airway cells stimulated by urban PM.

REFERENCES

1. Wardlaw T, Salama P, Johansson EW, Mason E. Pneumonia: the leading killer of children. *Lancet* 2006;368:1048-50.
2. Farha T, Thomson AH. The burden of pneumonia in children in the developed world. *Paediatr Respir Rev* 2005;6:76-82.
3. van der Poll T, Opal SM. Pathogenesis, treatment, and prevention of pneumococcal pneumonia. *Lancet* 2009;374:1543-56.
4. Wilson R, Dowling RB, Jackson AD. The biology of bacterial colonization and invasion of the respiratory mucosa. *Eur Respir J* 1996;9:1523-30.
5. Weidenmaier C, Peschel A. Teichoic acids and related cell-wall glycopolymers in gram-positive physiology and host interactions. *Nat Rev Microbiol* 2008;6:276-87.
6. Cundell DR, Gerard NP, Gerard C, Idanpaan-Heikkilä I, Tuomanen EI. Streptococcus pneumoniae anchor to activated human cells by the receptor for platelet-activating factor. *Nature* 1995;377:435-8.
7. Ishizuka S, Yamaya M, Suzuki T, Nakayama K, Kamanaka M, Ida S, et al. Acid exposure stimulates the adherence of Streptococcus pneumoniae to cultured human airway epithelial cells: effects on platelet-activating factor receptor expression. *Am J Respir Cell Mol Biol* 2001;24:459-68.
8. Rijnveld AW, Weijer S, Florquin S, Speelman P, Shimizu T, Ishii S, et al. Improved host defense against pneumococcal pneumonia in platelet-activating factor receptor-deficient mice. *J Infect Dis* 2004;189:711-6.
9. Nuorti JP, Butler JC, Farley MM, Harrison LH, McGeer A, Kolczak MS, et al. Cigarette smoking and invasive pneumococcal disease. Active Bacterial Core Surveillance Team. *N Engl J Med* 2000;342:681-9.
10. Barnett AG, Williams GM, Schwartz J, Neller AH, Best TL, Petroschovsky AL, et al. Air pollution and child respiratory health: a case-crossover study in Australia and New Zealand. *Am J Respir Crit Care Med* 2005;171:1272-8.
11. Neupane B, Jerrett M, Burnett RT, Marrie T, Arain A, Loebe M. Long-term exposure to ambient air pollution and risk of hospitalization with community-acquired pneumonia in older adults. *Am J Respir Crit Care Med* 2005;171:47-53.
12. Medina-Ramon M, Zanobetti A, Schwartz J. The effect of ozone and PM10 on hospital admissions for pneumonia and chronic obstructive pulmonary disease: a national multicity study. *Am J Epidemiol* 2006;163:579-88.
13. Cohen AJ, Ross Anderson H, Ostro B, Pandey KD, Krzyzanowski M, Kunzli N, et al. Urban air pollution. In: Ezzati M, Lopez AD, Rodgers A, Murray CL, editors. Comparative quantification of health risks: global and regional burden of disease attributable to selected major risk factors. Geneva: World Health Organization; 2004. p. 1353-433.
14. Donaldson K, Tran L, Jimenez LA, Duffin R, Newby DE, Mills N, et al. Combustion-derived nanoparticles: a review of their toxicology following inhalation exposure. *Part Fibre Toxicol* 2005;2:10.
15. Bunn HJ, Dinsdale D, Smith T, Grigg J. Ultrafine particles in alveolar macrophages from normal children. *Thorax* 2001;56:932-4.
16. Kocbach A, Johansen BV, Schwarze PE, Namork E. Analytical electron microscopy of combustion particles: a comparison of vehicle exhaust and residential wood smoke. *Sci Total Environ* 2005;346:231-43.
17. Donaldson K, Stone V, Borm PJ, Jimenez LA, Gilmour PS, Schins RP, et al. Oxidative stress and calcium signaling in the adverse effects of environmental particles (PM10). *Free Radic Biol Med* 2003;34:1369-82.
18. Calcebrini A, Meschini S, Marra M, Falzano L, Colone M, De Berardis B, et al. Fine environmental particulate engenders alterations in human lung epithelial A549 cells. *Environ Res* 2004;95:82-91.
19. Air quality in Europe. Available at: http://www.airqualitynow.eu/city_info/leicester/page2.php. Accessed December 31, 2010.
20. Arku RE, Vallarino J, Dionisio KL, Willis R, Choi H, Wilson JG, et al. Characterizing air pollution in two low-income neighborhoods in Accra, Ghana. *Sci Total Environ* 2008;402:217-31.
21. Brown DM, Hutchison L, Donaldson K, Stone V. The effects of PM10 particles and oxidative stress on macrophages and lung epithelial cells: modulating effects of calcium-signaling antagonists. *Am J Physiol Lung Cell Mol Physiol* 2007;292:L1444-51.
22. Hammerschmidt S, Wolff S, Hocke A, Rosseau S, Muller E, Rohde M. Illustration of pneumococcal polysaccharide capsule during adherence and invasion of epithelial cells. *Infect Immun* 2005;73:4653-67.
23. Okada N, Tatsuno I, Hanski E, Caparon M, Sasakawa C. Streptococcus pyogenes protein F promotes invasion of HeLa cells. *Microbiology* 1998;144:3079-86.
24. Adediji A, Weller TM, Ayres JG. Effect of PM10 on H. influenzae and S. pneumoniae. *Thorax* 2004;59:542-3.
25. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods* 1983;65:55-63.
26. LeVan TD, Dow SB, Chase PB, Bloom JW, Regan JW, Cunningham E, et al. Evidence for platelet-activating factor receptor subtypes on human polymorphonuclear leukocyte membranes. *Biochem Pharmacol* 1997;54:1007-12.
27. Casals-Stenzel J, Muacevic G, Weber KH. Pharmacological actions of WEB 2086, a new specific antagonist of platelet activating factor. *J Pharmacol Exp Ther* 1987;241:974-81.
28. Suzuki M, Thiem VD, Yanai H, Matsubayashi T, Yoshida LM, Tho LH, et al. Environmental tobacco smoking exposure is associated with an increased risk of

- hospitalized pneumonia among children under 5 years old in Vietnam. *Thorax* 2009;64:484-9.
29. Dherani M, Pope D, Mascarenhas M, Smith KR, Weber M, Bruce N. Indoor air pollution from unprocessed solid fuel use and pneumonia risk in children aged under five years: a systematic review and meta-analysis. *Bull World Health Organ* 2008;86:390-8.
 30. Sehlstedt M, Dove R, Boman C, Pagels J, Swietlicki E, Lundahl J, et al. Antioxidant airway responses following experimental exposure to wood smoke in man. *Part Fibre Toxicol* 2010;7:21.
 31. MacNee W, Donaldson K. Mechanism of lung injury caused by PM10 and ultra-fine particles with special reference to COPD. *Eur Respir J Suppl* 2003;40: 47s-51s.
 32. Mudway IS, Duggan ST, Venkataraman C, Habib G, Kelly FJ, Grigg J. Combustion of dried animal dung as biofuel results in the generation of highly redox active fine particulates. *Part Fibre Toxicol* 2005;2:6.
 33. Rhoden CR, Lawrence J, Godleski JJ, Gonzalez-Flecha B. N-acetylcysteine prevents lung inflammation after short-term inhalation exposure to concentrated ambient particles. *Toxicol Sci* 2004;79:296-303.
 34. Osornio-Vargas AR, Lindroos PM, Coin PG, Badgett A, Hernandez-Rodriguez NA, Bonner JC. Maximal PDGF-induced lung fibroblast chemotaxis requires PDGF receptor- α . *Am J Physiol* 1996;271:L93-9.
 35. Delfino RJ, Quintana PJ, Floro J, Gastanaga VM, Samimi BS, Kleinman MT, et al. Association of FEV1 in asthmatic children with personal and microenvironmental exposure to airborne particulate matter. *Environ Health Perspect* 2004;112: 932-41.
 36. Dionisio KL, Arku RE, Hughes AF, Vallarino J, Carmichael H, Spengler JD, et al. Air pollution in Accra neighborhoods: spatial, socioeconomic, and temporal patterns. *Environ Sci Technol* 2010;44:2270-6.
 37. Colbeck I, Nasir ZA, Ali Z. Characteristics of indoor/outdoor particulate pollution in urban and rural residential environment of Pakistan. *Indoor Air*;20:40-51.
 38. Kim YJ, Jung CY, Shin HW, Lee KL. Biomass smoke induced bronchial anthracofibrosis: presenting features and clinical course. *Respir Med* 2009;103:757-65.
 39. Isaacs KK, Schlesinger RB, Martonen TB. Three-dimensional computational fluid dynamics simulations of particle deposition in the tracheobronchial tree. *J Aerosol Med* 2006;19:344-52.
 40. Sigaud S, Goldsmith CA, Zhou H, Yang Z, Fedulov A, Imrich A, et al. Air pollution particles diminish bacterial clearance in the primed lungs of mice. *Toxicol Appl Pharmacol* 2007;223:1-9.
 41. Avadhanula V, Rodriguez CA, Devincenzo JP, Wang Y, Webby RJ, Ulett GC, et al. Respiratory viruses augment the adhesion of bacterial pathogens to respiratory epithelium in a viral species- and cell type-dependent manner. *J Virol* 2006;80: 1629-36.

METHODS

Glutathione assay

A total of 450 μL of supernatant from freeze-thawed A549 cells maintained on wet ice was acidified with 50 μL of 50% metaphosphoric acid (4°C) before the addition of 100 μL heptane (4°C). The acidified sample was then vortexed for 30 seconds before centrifugation at 13,000 rpm for 5 minutes at 4°C to remove the protein and lipid components. The supernatant was then removed from beneath the heptane interface and transferred to an amber HPLC vial. Glutathione concentration was determined by using reverse-phase HPLC with an EG&G amperometric electrochemical detector (Jones Chromatography, Hengoed, UK) operated at a 1000-mV electrode voltage, a 5-second time constant, and a cathodic output sensitivity of 0.2 milliamperes. A total of 20 μL of each sample and standard was injected by using a Gilson model 231 auto-sampler (Gilson Inc, Middleton, Wis; 4°C) onto a $10 \times 300\text{-nm}$, $5\text{-}\mu\text{m}$ C18 column that was eluted at a flow rate of 1.5 mL/min with 0.2 mmol/L $\text{K}_2\text{HPO}_4\text{-H}_3\text{PO}_4$ mobile phase containing 0.25 mmol/L octanesulfonic acid (pH 2.1). Sample concentrations were calculated according to standard curves of glutathione ranging from 0 to 50 $\mu\text{mol/L}$.

Transmission electron microscopy

A549 cells cultured on coverslips were cultured with PM_{10} (UK), then fixed in 2.5% glutaraldehyde in 0.1 mol/L sodium phosphate buffer (pH 7.3) and subjected to buffers, 2×5 minutes in 0.067 mol/L sodium phosphate buffer with 0.25 mol/L sucrose, 15 minutes in 1% osmium tetroxide in 0.13 mol/L Millonig phosphate buffer with 0.3 mol/L glucose (pH 7.3), 10 minutes

each in 10%, 50%, and 70% ethanol, 4×15 minutes in 100% ethanol, then 3 hours in TAAB medium resin (TAAB Laboratories Equipment Ltd, Aldermaston, UK). For each coverslip, 1 drop of TAAB resin was placed on a glass slide; the coverslip was placed cell side up on top of the drop of resin; a second drop of resin was placed on top of the coverslip; and a resin-filled BEEM capsule (Agar Scientific, Stansted, UK) was inverted, placed on top, and polymerized at 70°C overnight. The sample was removed and allowed to cool at room temperature. Each slide was placed on a 90°C hotplate for 30 seconds and the BEEM capsule pulled rapidly away from the coverslip. Each block was then cut into 2 sections, 1 of which was sectioned en face and the other re-embedded in an orientation to allow transverse sectioning. Ultrathin sections were cut by using a Reichert Ultracut E ultramicrotome (Reichert, Depew, NY). Sections were stained for 15 minutes in Reynold lead citrate. Examined images were recorded using a Hitachi H7600 transmission electron microscope (Hitachi High-Technologies Europe GmbH, Krefeld, Germany) fitted with a digital camera system (Advanced Microscopy Techniques Corp, Danvers, Mass).

Scanning electron microscopy

A549 cells were adhered to coverslips and cultured with PM_{10} (UK) and *S. pneumoniae* in medium. Cells were cryogenically fixed by plunge-freezing in liquid nitrogen slush. Frozen samples were vacuum transferred into a Gatan Alto 2500 cryopreparation chamber (Gatan Inc, Pleasanton, Calif), where ice was sublimed off the surface at -90°C for 3 minutes, coated with platinum for 30 seconds at -130°C , and transferred directly into a Quanta 3D FEG dual-beam scanning electron microscope (Fei Company, Hillsboro, Ore) via an airlock.

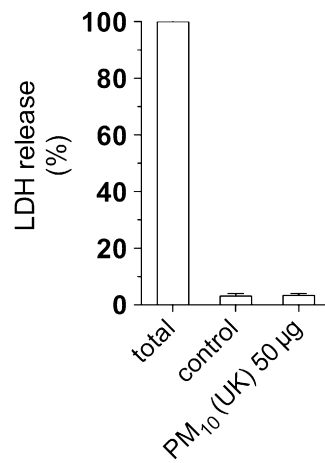


FIG E1. Lactate dehydrogenase (LDH) release from A549 cells cultured with PM₁₀ (UK). LDH release is indexed to maximum possible LDH release determined after cell lysis (total = 100%). Cells were cultured for 4 hours with either medium alone (control) or with PM₁₀ (UK) at 50 µg/mL. Data are given as means and SEMs (n = 5). LDH release is low from both control and PM-stimulated cells with no significant difference between control and PM₁₀ (UK)-exposed cells ($P = \text{NS}$ by t test control vs PM₁₀ treated cells).