Exposure to welding fumes and lower airway infection with *Streptococcus pneumoniae*

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Background: Welders are at increased risk of pneumococcal pneumonia. The mechanism for this association is not known. The capacity of pneumococci to adhere to and infect lower airway cells is mediated by host-expressed platelet-activating factor receptor (PAFR).

Objective: We sought to assess the effect of mild steel welding fumes (MS-WF) on PAFR-dependent pneumococcal adhesion and infection to human airway cells *in vitro* and on pneumococcal airway infection in a mouse model.

Methods: The oxidative potential of MS-WF was assessed by their capacity to reduce antioxidants *in vitro*. Pneumococcal adhesion and infection of A549, BEAS-2B, and primary human bronchial airway cells were assessed by means of quantitative bacterial culture and expressed as colony-forming units (CFU). After intranasal instillation of MS-WF, mice were infected with *Streptococcus pneumoniae*, and bronchoalveolar lavage fluid (BALF) and lung CFU values were determined. PAFR protein levels were assessed by using immunofluorescence and immunohistochemistry, and PAFR mRNA expression was assessed by using quantitative PCR. PAFR was blocked by CV-3988, and oxidative stress was attenuated by N-acetylcysteine.

Results: MS-WF exhibited high oxidative potential. In A549 and BEAS-2B cells MS-WF increased pneumococcal adhesion and infection and PAFR protein expression. Both CV-3988 and N-acetylcysteine reduced MS-WF–stimulated pneumococcal adhesion and infection of airway cells. MS-WF increased mouse lung PAFR mRNA expression and increased BALF and lung pneumococcal CFU values. In MS-WF–exposed mice CV-3988 reduced BALF CFU values.

Conclusions: Hypersusceptibility of welders to pneumococcal pneumonia is in part mediated by the capacity of welding fumes to increase PAFR-dependent pneumococcal adhesion and infection of lower airway cells. (J Allergy Clin Immunol 2015;■■■:■■■-■■■.)

Key words: *Occupational disease, welding fumes, platelet-activating factor receptor,* Streptococcus pneumoniae*, pneumonia, bacterial adhesion and infection*

Occupational data from England and Wales for 1970 to 1972 report there were 66 deaths among welders compared with 42 expected deaths.[1] Similar data for 1990 to 2000 suggest that excess deaths among welders are due to pneumonias other than bronchopneumonia, principally lobar pneumonia, and are present in other occupations associated with exposure to metal fumes.[2] Hypersusceptibility to pneumonia appears to be reversible because excess deaths are limited to welders of less than the normal retirement age,[2] and a recent United Kingdom (UK) case-control study found that hospital admissions for community-acquired pneumococcal pneumonia in working-age men were associated with occupational exposure to metal fumes in the past year but not in earlier periods.[3] Increased risk of pneumonia in welders has also been found outside the UK. For example, in a cohort of more than 30,000 Swedish construction workers with exposure to metal fumes, Toren et al[4] reported that mortality from lobar pneumonia was 3.7-fold higher and mortality from pneumococcal pneumonia was 5.8-fold higher relative to their peers. By contrast, deaths from pneumonia in retired metal workers were only marginally increased.[4] Although these findings suggest that inhalation of welding fumes (WF) increases the risk of pneumococcal infection, the high prevalence of other exposures in welders associated with increased risk of pneumococcal disease, such as smoking,[5,6] and the lack of a biologically plausible mechanism result in uncertainties about causality. However, animal studies reporting that WF impair pulmonary clearance of *Listeria monocytogenes*[7-9] suggest that WF have the potential to adversely affect the pulmonary innate immune system.

Adherence of pneumococci to lower airway cells is a first step in the development of airway infection leading to pneumonia.[10] For *Streptococcus pneumoniae* (and other phosphorylcholine-expressing bacteria, such as nontypeable *Haemophilus influenzae*[11] and *Acinetobacter* species[12]), adhesion and infection of lower airway cells is facilitated by an interaction between bacterial phosphorylcholine and the platelet-activating factor receptor (PAFR; 10-alkyl-2-acetyl-glycerophosphocholine PAF) expressed on host cells.[13] Because previous studies report that inhaled toxins,

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Abbreviations used
BALF: Bronchoalveolar lavage fluid
CFU: Colony-forming units
IPD: Invasive pneumococcal disease
LDH: Lactate dehydrogenase
MS-WF: Mild steel welding fumes
NAC: N-acetylcysteine
OP: Oxidative potential
PAFR: Platelet-activating factor receptor
PM: Particulate matter
StS-WF: Stainless steel welding fumes
UK: United Kingdom
WF: Welding fumes

including fossil fuel–derived particulate matter (PM) and cigarette smoke,[14,15] through induction of oxidative stress, upregulate PAFR-dependent adhesion of pneumococci to airway epithelial cells, we hypothesized that hypersusceptibility to pneumonia in welders is mediated through PAFR-dependent pneumococcal adhesion. Therefore in this study we sought to assess the oxidative potential (OP) of mild steel welding fumes (MS-WF), the effect of MS-WF on PAFR-dependent pneumococcal adhesion and infection in human lower airway cells *in vitro*, and PAFR-dependent pneumococcal airway infection in a mouse model. We also assessed PAFR in stored lung tissue from a study in which mice were exposed to aerosolized stainless steel welding fumes (StS-WF) and from a study of particles in welders' lungs.[16]

METHODS

WF: Generation and composition

MS-WF were a gift from the Welding Institute (Cambridge, UK). MS-WF were obtained by using a standardized method in accordance with the International Standard 15011-1:2009, as previously described.[17] Briefly, manual metal arc welding electrodes (mild steel E7018 basic type) were run to produce a weld bead inside a fume collection system. MS-WF with a mode particle diameter of 6.8 μm[18] were extracted through the hood on top of the box, collected on a filter paper, removed by brushing, and stored in airtight glass containers. The composition of MS-WF was determined after digestion in nitric/hydrochloric acid in a high-temperature, closed-vessel, microwave-assisted dissolution system. Analysis was done by using inductively coupled plasma–atomic emission spectroscopy. Before use, MS-WF were suspended in PBS.

WF: OP

The OP of MS-WF was determined based on their ability to oxidize antioxidants from a validated *in vitro* respiratory tract lining fluid model containing equimolar (200 μmol/L) and physiologically relevant concentrations of ascorbate, urate, and glutathione.[19] Incubations were performed with particle suspensions at a final concentration of 50 μg/mL for 4 hours at 37°C (pH 7.4) in parallel to particle-free and PM controls (an oxidatively inert carbon black [M120] and an oxidatively active urban PM [NIST1648a]). At the end of this period, particles were removed by means of centrifugation (13,000 rpm at 4°C), and samples were acidified with metaphosphoric acid (final concentration 5%) before determination of the remaining antioxidant concentrations by using reverse-phase HPLC with electrochemical detection (for ascorbate) and the glutathione disulphide-reductase-5, 5′-dithio-bis (2-nitrobenzoic acid) recycling assay (for glutathione).[19] OP was determined based on the percentage loss of ascorbate and glutathione over the 4-hour incubation period relative to a 4-hour particle-free control (reflecting background auto-oxidation rates). Under these conditions, urate losses are not significant.[16,17] The percentage loss of ascorbate and glutathione over the 4-hour incubation was then normalized to the particle concentration used in the respiratory tract lining fluid assay (50 μg/mL) to generate 2 separate measures of OP: glutathione-dependent OP ($OP^{glutathione}$) per microgram and ascorbate-dependent OP ($OP^{ascorbate}$) per microgram. In addition, an aggregate sum of the 2 measures was calculated (OP^{total} per microgram),[20] previous work having shown that ascorbate and glutathione oxidation is sensitive to different panels of oxidants.[16,17]

Pneumococcal adhesion and infection: Human airway cells

A549 cells, a type II pneumocyte cell line (Sigma-Aldrich, Poole, UK), were maintained in Dulbecco modified Eagle medium supplemented with FBS, L-glutamine, and antibiotics (Lonza, Basel, Switzerland). Passage number was less than 20. BEAS-2B, a bronchial epithelial cell line, was a gift from Dr Nicolas Mercardo (National Heart and Lung Institute, Imperial College London, London, UK). BEAS-2B cells were maintained in RPMI-1640 medium containing HEPES (Life Technologies, Warrington, UK) supplemented with FBS L-glutamine and antibiotics. Passage number was less than 20.

Cell viability was assessed by using the lactate dehydrogenase (LDH) assay (Sigma-Aldrich), according to the manufacturer's instructions. Cells treated with distilled water (indicating 100% LDH release) were used as a positive control. Primary human bronchial epithelial cells (purchased from Promocell, Heidelberg, Germany; lot no. 4032402) were maintained according to the manufacturer's instructions. Passage number was less than 4. The type 2 *S pneumoniae* encapsulated strain D39 was purchased from the National Collection of Type Cultures (NCTC 7466; Central Public Health Laboratory, London, UK) and grown in liquid culture brain-heart infusion broth (Oxoid, Basingstoke, UK) to the midlogarithmic phase (OD_{600} = 0.4-0.6) before use.

Pneumococcal adhesion and infection, and infection alone of airway cells were assessed by using a standard *in vitro* assay.[14,15] Briefly, airway epithelial cells were cultured with MS-WF for 2 hours, washed, and infected with *S pneumoniae* at a multiplicity of infection of 100 for 2 hours to assess the combination of pneumococcal adhesion and infection of cells. Cells were then vigorously washed, detached, and lysed with sterile distilled water. Serial dilutions of the samples were plated on brain-heart infusion agar containing 5% horse blood (Oxoid), and colony-forming units (CFU) per milliliter were assessed. In this assay CFU values after cell lysis reflect both pneumococci attached to the surfaces of airway cells (ie, the adherent fraction) and pneumococci that penetrate into cells (ie, the infective fraction). The adherent fraction was first killed with gentamicin (200 mg/mL) and penicillin G (10 mg/mL), to assess the infective fraction alone. Intracellular pneumococci that were protected from antibiotics were recovered by means of cell lysis with ice-cold sterile water, and CFU values were determined.[15] The functional role of PAFR was assessed by adding a specific PAFR blocker, (RS)-2-methoxy-3-(octadecylcarbamoyloxy)-propyl2-(3-thiazolio) ethylphosphate (CV-3988),[21] at a final concentration of 20 μmol/L. The role of oxidative stress was assessed by adding the thiol antioxidant N-acetylcysteine (NAC; Sigma-Aldrich)[22] at a final concentration of 5 mmol/L at the same time as MS-WF.

Pneumococcal infection: Mouse model

Four- to 6-week-old female CD1 mice (Charles River, Welwyn Garden City, UK) were exposed to MS-WF in 50 μL of PBS (administered either as a single 600-μg dose or as divided doses) through intranasal installation after achievement of isoflurane anesthesia. Twenty-four hours after instillation of MS-WF, animals were intranasally infected with 5×10^6 *S pneumoniae* strain D39 in 50 μL of PBS. Animals were killed at 24 hours after pneumococcal infection by using a pentobarbital overdose. Pneumococcal CFU values in bronchoalveolar lavage fluid (BALF), lung tissue (done after BAL), and blood were assessed by plating serial dilutions on brain-heart infusion agar containing 5% horse blood (Oxoid). Mouse experiments were approved by University College London's Biological Services Ethical Committee under UK Home Office Project License PPL70/6510 and performed according to UK national guidelines for animal use and care under UK Home Office license in accordance with European Union Directive 2010/63/EU. Animals received 30 μL of 5 mg/kg of the PAFR blocker CV-3988 (Sigma-Aldrich) administered by means of tail vein injection 1 hour before pneumococcal infection to assess the effect of blocking PAFR.