*Haemophilus influenzae* from COPD Patients with Exacerbation Induce More Inflammation than Colonizers

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Online Data Supplement
Methods

COPD Patients

Inclusion criteria for COPD patients were the presence of chronic bronchitis (E1), absence of other lung disease on the basis of clinical assessment, absence of immunosuppressive or other life-threatening disorders, and willingness to attend monthly clinic visits. Patients were seen at the COPD Study Clinic monthly and whenever they had symptoms suggestive of an exacerbation. At each visit, clinical information, expectorated sputum, and serum samples were obtained. Patients were questioned about the status of their chronic respiratory symptoms (dyspnea, cough, and sputum production, viscosity, and purulence) and were graded as 1 (at the usual level), 2 (somewhat worse than usual), or 3 (much worse than usual). A grade 2 worsening of two or more symptoms or grade 3 worsening of one or more symptoms prompted a clinical assessment of the cause. If the patient had a fever (temperature > 38.3°C), appeared ill, or had signs of consolidation on lung examination, chest x-ray films were obtained. The patient was considered to have a COPD exacerbation if other causes of symptom worsening, such as pneumonia, upper respiratory infection, or congestive heart failure, were excluded by 1 of 2 examiners (SS, TM), and this determination occurred before the results of sputum cultures were available (E2, E3).

Bacterial Isolation

Personnel who processed sputum samples were unaware of the clinical status of patients. Isolates were confirmed as *H. influenzae* by growth requirement for hemin and nicotinamide adenine dinucleotide (X and V factors) and by the absence of porphyrin. *H. influenzae* isolates were subjected to molecular typing by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and pulsed-field gel electrophoresis as described previously (E3-E5). A new strain was one that had not been isolated from sputum samples previously obtained from an individual patient. Each *H. influenzae* strain was tested with two homologous patient sera to determine whether serum antibodies to the infecting strain developed after an exacerbation as described previously (E6). The preexacerbation serum was obtained at a clinic visit before the
exacerbation visit and the postexacerbation serum was obtained at least 4 weeks after the exacerbation visit. A new antibody response was defined as the development of new serum bactericidal antibodies to the homologous infecting strain and new antibodies by whole cell enzyme-linked immunosorbent assay (ELISA) compared to serum obtained one month prior to the exacerbation (E3, E6).

**Bacterial Preparation**

After isolation from sputum samples, bacteria were stored as glycerol stocks. *H. influenzae* were grown on chocolate blood agar supplemented with 1% Isovitalex (BD Scientific, Sparks, MD) or in brain-heart infusion broth supplemented with hemin and NAD (E7). In separate experiments, growth rates of all bacterial isolates were determined using an initial 1 ml of bacteria with an OD$_{600}$ of 0.150 diluted in 24 ml of supplemented brain heart infusion broth. Bacteria were then incubated in a shaker at 37°C and growth curves were plotted from hourly measurements of absorbance at 600 nm.

**Mouse Airway Infection Model**

*H. influenzae* was incorporated into and mixed with amorphous agar particles (ranging in size from 30-200 µm) producing a suspension containing bacteria at approximately $10^{8-10}$ CFU/ml. Six week-old, C57BL/6J mice (Taconic, Germantown, NY) underwent orotracheal intubation with a 24-gauge intravenous catheter and immediate airway injection of 50 µl of an agar and bacteria suspension at 37°C (followed by 100 µl of air). After 24 h of infection, mice were anesthetized and then euthanized by cervical dislocation, the lungs were exposed, and the pulmonary vascular system flushed via the right ventricle with sterile saline. Airway leukocytes were isolated by bronchoalveolar lavage (BAL) of the left lung with 0.5 ml aliquots of PBS followed by quantitation of total leukocyte counts using a hemocytometer and differential counts using modified Wright's staining of cytospin preparations (E8). The four lobes of the right lung were ligated at their roots and removed under sterile conditions. For chemokine quantitation, homogenates from two lobes of the right lung were tested for mouse keratinocyte chemoattractant (KC) and macrophage inflammatory protein-2 (MIP-2) levels using quantitative
sandwich enzyme-linked immunoassay kits (R&D Systems, Minneapolis, MN) (E7). According to the manufacturer, the sensitivity of these assay systems for KC is <2.0 pg/ml and for MIP-2 is <1.5 pg/ml. For bacterial quantitation, homogenates from two lobes of the right lung underwent serial dilution followed by aseptic inoculation onto solid agar media for culture and quantification of lung bacterial load (E7, E8). Preliminary experiments verified a homogeneous bacterial load in all segments of both lungs in our animal model.

**Biofilm Formation Assay**

After bacterial incubation for 96 h, attached biofilms were stained with 1% crystal violet, washed, and dissolved in ethanol. Absorbance of dissolved biofilms at 570 nm for 6 samples per isolate was measured using a VERSAmax™ microplate reader (Molecular Devices, Sunnyvale, CA).

**Airway Epithelial Cell Isolation and Culture**

Epithelial cells were isolated from tracheal and bronchial mucosa by enzymatic dissociation and cultured in Laboratory of Human Carcinogenesis (LHC)-8e medium on plates coated with collagen and albumin for study up to passage 10 as described previously (E9, E10). Multiple donors supplied epithelial cells for replicate experiments to assure that differences were not attributable to responses in cells from one individual. For epithelial cell activation, bacteria were incubated in 100 µg/ml gentamicin for 30 minutes, and then 10⁸-10⁹ colony forming units (CFU)/ml (500-5,000 CFU/epithelial cell) of killed bacteria was incubated with epithelial cells in culture media for 1-24 hours.

**Bacterial Adherence Assay**

Bacterial adherence to hTBE cell monolayers at confluence on 96-well tissue cultures plates was quantified by modification of an assay described previously (E7). Bacteria were inoculated onto epithelial cell monolayers in antibiotic-free media, and plates were centrifuged at 165 x g for 5 min to facilitate bacteria-epithelial cell contact. After incubation at 37°C for 30 min, monolayers were washed and then lysed by addition of one volume of 2% saponin for 10
minutes combined with scraping and mixing. Serial dilutions were plated for bacterial quantitation.

**ICAM-1 and IL-8 Expression**

ICAM-1 protein levels on the surface of hTBE cell monolayers at confluence on 96-well tissue culture plates were determined by an enzyme-linked colorimetric immunoassay as described previously (E7, E10-E12). IL-8 concentrations in cell culture media were determined using a commercial sandwich enzyme-linked immunoassay kit (R&D Systems). According to the manufacturer, the sensitivity of this assay system for IL-8 is <10 pg/ml.

**Signaling Pathway Inhibition**

For assessment of nuclear factor-κB (NF-κB)-dependent signaling, hTBE cells were infected for 48 h prior to bacterial infection with one of the following recombinant adenoviral vectors: 1) Ad-IκBαDN that expresses a dominant negative mutant form of IκBα by substitution of alanines for serine-32 and -36 (a gift from D. Brenner, Univ. of North Carolina)(E13); 2) Ad5cmvEGFP expressing the control transgene green fluorescence protein (GFP)(provided by the University of Iowa Gene Therapy Center, http://genetherapy.genetics.uiowa.edu/). Recombinant adenoviruses were propagated and titered in human 293 cells (CRL#1573, American Type Culture Collection) as previously described (E10-E12). Epithelial cell expression of GFP was detected using inverted fluorescence photomicroscopy (model DM IRB, Leica Microsystems, Wetzlar, Germany) as described previously (E10). Electronically digitized images were acquired using a CCD camera (Model DXC-970MB; Sony Corporation, Montvale, NJ) interfaced with ImagePro Plus software (version 4.1, Media Cybernetics, Silver Spring, MD). For assessment of p38-dependent signaling, hTBE cells were pretreated for 1 h with either control DMSO carrier or the p38 mitogen-activated protein (MAP) kinase inhibitor SB203580 (Biosource International, Carmarillo, CA).

**NF-κB Activation Assay**

NF-κB activation was determined by infection of hTBE cell monolayers at multiplicity of infection (MOI) 1 for 24 h with the recombinant adenoviral vector Ad-NFκBluc that expresses a luciferase gene driven by four tandem NF-κB sites (a gift from P. McCray, Univ. of Iowa)(E14).
Epithelial cells were then incubated for an additional 24 h without or with equivalent inoculums of *H. influenzae* isolates from patients with COPD. *Photinus pyralis* luciferase activity was determined as previously described or using a commercial luciferase reporter assay kit (Promega, Madison, WI)(E11, E15, E16).

**MAP kinase Activation Analysis**

Phosphorylated and total MAP kinase protein levels were assessed by immunoblot analysis as described previously (E11, E12). Primary antibodies used for immunoblot analysis included rabbit monoclonal IgG 3D7 that recognizes human p38α, p38β, and p38γ phosphorylated at threonine 180 and tyrosine 182 (Cell Signaling Technology, Beverly, MA), mouse monoclonal IgG 2F11 that recognizes human total p38α and p38β (Biosource), rabbit polyclonal immunoglobulin 9101 that recognizes human ERK1 and ERK2 phosphorylated at threonine 202 and tyrosine 204 (Cell Signaling), or rabbit polyclonal immunoglobulin C-14 that recognizes human total ERK1 and ERK2 (Santa Cruz Biotechnology, Santa Cruz, CA). For these experiments, whole cell protein extracts were prepared by lysis of cell monolayers in 50 mM Tris, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, a protease inhibitor cocktail (Roche Bioscience, Palo Alto, CA), and a phosphatase inhibitor panel (Calbiochem, San Diego, CA). Equal amounts of cell protein were subjected to SDS-PAGE in 7.5% polyacrylamide. Resolved proteins were electrophoretically transferred to nitrocellulose membranes (Hybond™, Amersham Biosciences, Piscataway, NJ), exposed to 5% nonfat milk in Tris-buffered saline with 0.1% Tween-20 to block nonspecific antigens, and then incubated with antibody against a specific cellular protein. Primary antibody binding was detected using goat antirabbit IgG or goat antimouse IgG conjugated to horseradish peroxidase (Santa Cruz) and an enhanced chemiluminescence detection system (Amersham). Repробing of membranes with a different primary antibody was done after washing in Restore™ buffer (Pierce, Rockford, IL) for 15 min at 37°C. Band densitometry was performed using a Fluor-S™ Max MultiImager interfaced with Quantity One software version 4.4.0 (Bio-Rad Laboratories, Hercules, CA).
References


