Airway Inflammation and Bronchial Bacterial Colonization in Chronic Obstructive Pulmonary Disease

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Online data supplement

Study Subjects. The study protocol was approved by the VA WNY Healthcare System institutional review board. Three groups of volunteer subjects were enrolled. Inclusion criteria for Group 1, ex-smokers with COPD included evidence of obstructive airways disease on spirometry; ≥20 pack yrs of cigarette smoking but cessation of smoking at least 1 year prior to enrollment; clinical exclusion of other lung disease; chest x-ray that was either normal or had findings compatible with COPD; <15% bronchodilator response with inhaled albuterol on spirometry; no antibiotic or systemic steroid exposure in the 4 weeks preceding enrollment; and no history of cough syncope. A forced expiratory volume in 1 second (FEV_1) of <35% predicted, hypercapnia or other systemic disease that made bronchoscopy risky were exclusion criteria. Inclusion criteria for Group 2, ex-smokers without COPD, were the same as for Group 1 except for absence of lung disease by clinical evaluation, chest x-ray and spirometry. Inclusion criteria for Group 3, healthy non-smokers, were the same as Group 2 except they were life-long non-smokers, defined as no tobacco smoke exposure or remote exposure of <5 pack yrs.

BAL processing. The lavage fluid was placed on ice and processed within 30 minutes. Mucus was removed by filtering the lavage fluid through sterile gauze. Quantitative cultures were performed on the BAL fluid to determine frequency of colonization by potentially pathogenic bacteria (PPB). PPB included *Haemophilus* spp., *Streptococcus pneumoniae, Moraxella catarrhalis, Staphylococcus aureus, Pseudomonas aeruginosa*, and gram-negative enteric bacteria [E1]. A 1 ml aliquot of the BAL fluid was serially diluted from 1:10 to 1:100,000 with normal saline and dilutions were plated on chocolate agar, blood agar and MacConkey agar plates. Bacterial colonies isolated were counted,

and species identification was done by standard methods. A threshold concentration of 100 (10^2) colony forming units/ml was used to define significant growth of a PPB [E2].

Cytokine analysis Cytokine concentrations were determined by multianalyte microsphere assay, analyzed by flow cytometry [E3]. Capture and detection antibody pairs directed against different noncompeting epitopes of their respective cytokine and recombinant protein standards for IL-1β, TNF-α, IL-8, IL-12 (R&D Systems, Minneapolis, MN) and IL-10 (BD Pharmingen, San Diego, CA) were covalently coupled to Multi-Analyte carboxylated microspheres (Luminex Corp., Austin, TX), each set of which has a unique proportion of red and orange fluorescent dyes, through free amino groups with carbodiimide and N-hydroxysuccinimide. Cytokine capture antibodies were each coupled to a different microsphere bead set. A mixture of water-soluble 24 mM 1-Ethyl-3-(3 Dimethylaminopropyl)-Carbodiimide Hydrochloride (EDC) and 20 mM N-hydroxysulfosuccinimide (Sulfo-NHS) (Pierce Biotechnology, Rockford, IL) was used to activate free carboxyl groups on the beads. After activation, beads were washed in 50 mM 2-(N-morpholino) ethanesulfonic acid (MES, Pierce) and the antibody to be coupled, dialyzed into MES, was immediately added (250 ug/ml). After overnight rotation at ambient temperature, the beads were washed and resuspended in PBS-TBN (phosphate buffered saline, pH 7.4, containing 0.02% Tween 20, 0.1% bovine serum albumin, and 0.02% sodium azide, (Sigma Chemical Co., St. Louis, MO) at 8 x 10^6/ml.

Multiplexed assays were performed in 96-well microtiter plates (Multiscreen HV plates, Millipore, Billerica, MA) with PVDF membranes, prewetted and washed with PBS-TBN, using a Tecan Genesis liquid handling robot (Research Triangle Park, NC) for

all manipulations of the microtiter plate. Bead sets coated with capture antibody were diluted in PBS-TBN and pooled. 1000 beads from each set were added per well. Recombinant protein standards were titrated from 33,333 to 0.56 pg/ml using 3-fold dilutions in PBS-TBN. Samples were diluted 4 fold in PBS-TBN and standards and samples were added in 50 ul volumes to wells containing beads. Plates were incubated at ambient temperature for 20 minutes on a rocker, washed twice with PBS using a vacuum manifold to aspirate. Biotinylated detection antibodies for each cytokine, diluted in PBS-TBN, were added in 50 ul volumes and incubated for 20 min. before the beads were washed with PBS. The appropriate detection antibody concentration, defined as the lowest titer that still saturated the standard curve, was predetermined for each detection antibody and capture bead set. 50 ul of phycoerythrin-conjugated streptavidin (Caltag Laboratories, Burlingame, CA) was added to each well and the plates were incubated and washed as before. The beads were resuspended in 150 ul of PBS-TBN and analyzed by flow cytometry (Luminex 100, Luminex Corp).

Samples were measured in duplicate and blank values were subtracted from all readings. A log-log regression was calculated (Microsoft Excel, Microsoft, Redmond, WA) using the bead mean fluorescence intensity (MFI) values for each concentration of recombinant protein standard. Points below detection limits or above saturation were excluded from the curve. Sample cytokine concentrations were calculated from MFIs of beads by interpolating the resulting best fit line. Samples with values above the standard curve were further diluted and reanalyzed.

Active MMP-9 measurement. Semiquantitative measurement of MMP-9 activity by zymography was performed as described by Russell et al [E4]. Sample buffer (250mM Tris, pH 6.8, 4% sucrose, 0.18% bromophenol blue, 10% SDS) was added to aliquots of un-concentrated, and if necessary, concentrated BAL samples. These samples were electrophoresed in 10% sodium dodecyl sulfate - polyacrylamide gels containing 0.1% gelatin. The gel was washed three times for 15 minutes in wash buffer 1(20 mM Tris buffer, ph 7.8, 2.5% Triton X-100) followed by 2 washes for 15 minutes each in wash buffer 2 (20 mM Tris, pH 7.8, 1% Triton X-100, 10 mM $CaCl_2$). The gel was then incubated overnight at 37°C in wash buffer 2. The gel was stained with 1% coomassie blue in 45% methanol followed by destaining in 25% methanol with 7.5% acetic acid to visualize bands of gelatin lysis (negative staining). Bands at 82 kDa, which represent active MMP-9, were analyzed by densitometry (AlphaEase FC software, Alpha Innotech, San Leandro, CA). Linear standard curves were generated by using known quantities of purified MMP-9 (Triple Point Biologics, Forest Grove, OR) and the amount of active MMP-9 in the BAL samples was estimated by interpolation on these curves and expressed as arbitrary densitometric units.

Reference List

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