Corticosteroid therapy and airflow obstruction influence the bronchial microbiome, which is distinct from that of bronchoalveolar lavage in asthmatic airways

Darcy R. Denner, PhD,[a] Naseer Sangwan, PhD,[b] Julia B. Becker, MD,[a] D. Kyle Hogarth, MD,[a] Justin Oldham, MD,[a] Jamee Castillo, MD,[a] Anne I. Sperling, PhD,[a] Julian Solway, MD,[a] Edward T. Naureckas, MD,[a] Jack A. Gilbert, PhD,[b,c,d,e] and Steven R. White, MD[a] *Chicago and Argonne, Ill, and Woods Hole, Mass*

Background: The lung has a diverse microbiome that is modest in biomass. This microbiome differs in asthmatic patients compared with control subjects, but the effects of clinical characteristics on the microbial community composition and structure are not clear.

Objectives: We examined whether the composition and structure of the lower airway microbiome correlated with clinical characteristics of chronic persistent asthma, including airflow obstruction, use of corticosteroid medications, and presence of airway eosinophilia.

Methods: DNA was extracted from endobronchial brushings and bronchoalveolar lavage fluid collected from 39 asthmatic patients and 19 control subjects, along with negative control samples. 16S rRNA V4 amplicon sequencing was used to compare the relative abundance of bacterial genera with clinical characteristics.

Results: Differential feature selection analysis revealed significant differences in microbial diversity between brush and lavage samples from asthmatic patients and control subjects. *Lactobacillus*, *Pseudomonas*, and *Rickettsia* species were significantly enriched in samples from asthmatic patients, whereas *Prevotella*, *Streptococcus*, and *Veillonella* species were enriched in brush samples from control subjects. Generalized linear models on brush samples demonstrated oral corticosteroid use as an important factor affecting the relative abundance of the taxa that were significantly enriched in asthmatic patients. In addition, bacterial α-diversity in brush samples from asthmatic patients was correlated with FEV_1 and the proportion of lavage eosinophils.

Conclusion: The diversity and composition of the bronchial airway microbiome of asthmatic patients is distinct from that of nonasthmatic control subjects and influenced by worsening airflow obstruction and corticosteroid use. (J Allergy Clin Immunol 2015;■■■:■■■-■■■.)

Key words: *Asthma, microbiome, corticosteroids, FEV_1, bacteria, 16S ribosomal RNA*

Formerly thought to be sterile,[1,2] it is now clear that the lung is colonized by microbes from early infancy[3] and exposed continuously to air, as well as nasal, oropharyngeal, and gastrointestinal tract secretions. Although normally low in biomass compared with other body sites, such as the gastrointestinal tract, the ecology of the lung microbiome is diverse and complex,[2,4] and ecologic dynamics of the microbial community rather than just the presence of any individual species might be an important component of disease pathogenesis.

Previous investigations suggest that the lung microbiome might contribute to the pathogenesis of asthma. Both bacterial exposure and greater diversity of environmental microbial exposures in early childhood diminish the risk of subsequent asthma or allergy.[5,6] Commensal gastrointestinal microbiota can influence the development of atopy and asthma.[7] Infants whose upper airways were colonized with select organisms had an increased risk for asthma later in life.[3] Treatment of asthmatic patients with macrolide antibiotics might provide symptomatic relief for selected patients, although recent trials dispute this.[8-13]

Recent reports suggest the microbiome in the lower airways might be different in patients with asthma. Hilty et al[14] demonstrated with endobronchial brushes that the genus *Haemophilus* had a greater relative abundance and the genus *Prevotella* had a reduced relative abundance in the bronchi of adults with either asthma or chronic obstructive pulmonary disease compared with control subjects. Marri et al[15] demonstrated that 3 major phyla, Firmicutes, Actinobacteria, and Proteobacteria, accounted for more than 90% of total 16S rRNA sequences in the sputum of patients with mild asthma, and again, Proteobacteria were significantly enriched compared with microbial communities in the sputum of control subjects. Goleva et al[16] demonstrated that bronchoalveolar lavage (BAL) fluid of control subjects and patients with corticosteroid-resistant or corticosteroid-sensitive asthma had significantly different relative abundances of many bacterial

From [a]the Section of Pulmonary and Critical Care Medicine, Department of Medicine, [c]the Departments of Ecology & Evolution and Surgery, and [d]the Institute for Genetics, Genomics, and Systems Biology, University of Chicago; [b]the Biosciences Division (BIO), Argonne National Laboratory, Argonne; and [e]the Marine Biological Laboratory, Woods Hole.

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Corresponding author: Steven R. White, MD, Section of Pulmonary and Critical Care Medicine, University of Chicago, 5841 S Maryland Ave, MC6076, Chicago, IL 60637. E-mail: swhite@bsd.uchicago.edu.

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Abbreviations used
BAL: Bronchoalveolar lavage
EB: Endobronchial brushing
FDR: False discovery rate
GLM: Generalized linear regression model
ICS: Inhaled corticosteroid
OCS: Oral corticosteroid
OTU: Operational taxonomic unit

genera. Finally, using endobronchial brush samples previously collected from the Macrolides in Asthma study, Huang et al[17] demonstrated greater bacterial diversity in samples from asthmatic patients compared with those from healthy control subjects, which correlated with bronchial hyperresponsiveness.

These data suggest that the lower airway microbiome might differ in asthmatic patients. However, differences in sample collection and location within the lung, phenotypes of asthmatic patients, and differing use of corticosteroids are likely significant confounders that need to be considered to identify the microbial biomarkers of clinically relevant characteristics in asthmatic patients.

Our asthma clinical research program has been collecting lower airway samples from both carefully characterized asthmatic patients and control subjects.[18,19] To overcome previous sampling inconsistencies, we decided to elucidate and model the variability in the microbiome of different lung regions, specifically the bronchial (endobronchial brushing [EB] samples) and small (BAL samples) airways. We show significant differences in the relative abundance of bacterial taxa between samples from asthmatic patients and those from control subjects and between EB and BAL samples, and we demonstrate that the asthmatic EB microbiome correlates with the degree of airflow obstruction. In addition, we highlight anatomic localization and corticosteroid use as important factors influencing the relative abundance patterns of differentially abundant taxa. Our data, combined with those of previous studies, help set the stage for longitudinal studies that can answer important questions about the role of the lower airway microbiome in asthmatic patients.

METHODS

Subjects

This is a cross-sectional retrospective study. Adult asthmatic patients and control subjects were recruited from among the participants in previous asthma genetic and airway biology studies that were originally conducted from 2011 to 2013. Approval for the retrospective use of samples generated from these subjects was obtained from the Institutional Review Board at the University of Chicago. All subjects provided written informed consent at the time of recruitment. Additional information, including details of negative brush and reagent control sample collection, is provided in the Methods section in this article's Online Repository at www.jacionline.org.

Bronchoscopy was done by using standard methods and conscious sedation. Because of the nature of this study with retrospective identification of subjects, no oral or nasal control samples were available for microbial analysis. Sample collection at bronchoscopy is described in detail in the Methods section in this article's Online Repository.

Sample processing

DNA was extracted by using methods detailed in the Methods section in this article's Online Repository. Primers specific for the V4 region (515-806 bp) of the 16S rRNA encoding gene were used to generate amplicons. Samples with sufficiently high DNA loading after amplification were sequenced in a paired-end 150 bp run by using the Illumina MiSeq (Illumina, San Diego, Calif) at the National Laboratory for High-Throughput Genome Analysis Core at Argonne National Laboratory (Argonne, Ill). Paired-end reads were quality trimmed and processed for operational taxonomic unit (OTU) picking by using the UPARSE[20] pipeline set at 97% sequence identity cutoff. Taxonomic status was assigned to high-quality OTUs (<1% incorrect bases) by using "parallel assign taxonomy" script from QIIME software.[21] Multiple sequence alignment and phylogenetic reconstruction were performed with PyNast and FastTree.[21] The Phyloseq package[22] was used for ordination and α- and β-diversity analysis.

Statistical analysis

By using the Phyloseq package,[22] the OTU matrix was processed to remove OTUs containing less than 5 reads to reduce the PCR and sequencing-based bias; then the OTU table was rarified to the minimum numbers of reads present in the smallest library.

For ANOVA and *P* value correction, we performed *post hoc* test using the Tukey-Kramer test with effect size of "eta squared" and corrected for multiple tests by using the method of Benjamini and Hochberg. MetagenomeSeq[23] was used to identify the differentially abundant taxons across groups. Clinical data are expressed as means ± SEMs. Two-group analysis was performed with Welch *t* tests, followed by the Story false discovery rate (FDR) correction. We then used a generalized linear regression model (GLM) to examine the contribution of patients' demographic data on the abundance patterns of the differentially abundant bacterial genera.

RESULTS

In this study we used specimens collected from 3 asthmatic patients and 19 control subjects for lung microbiome evaluation. Study subjects' characteristics are summarized in Table I. As expected, asthmatic patients had a lower FEV_1 compared with control subjects ($P = .001$), a higher concentration of exhaled nitric oxide ($P = .05$), and a larger percentage of eosinophils in BAL fluid ($P = .001$). There were no identified differences based on sex, ancestry, or the proportion of subjects with a serum IgE level of greater than 100 U/mL, although asthmatic patients were significantly older than the control population (44.2 vs 34.3 years old, $P < .004$; Table I).

Airway microbial community in EB versus BAL samples

We sequenced bacterial 16S rRNA of EB and BAL samples from 39 asthmatic patients and 19 control subjects, 5 negative brush samples, and 7 negative reagent samples. A total of 1.1 million 16S rRNA V4 amplicon sequence reads (average sample depth, 14,918) were generated, which, after quality control (<0.1% incorrect bases), were clustered into 2,134 OTUs. By using a strict quality control, OTUs (n = 403) with variance of greater than 0.000001 were kept for further analysis.

By using the Greengenes database, 38 phyla were identified in our data set; of these, 6 (Firmicutes, Proteobacteria, Bacteroides, Fusobacteria, Acidobacteria, and Actinobacteria) accounted for 85.9% of all sequences, unclassified sequences accounted for 9.1% of sequences, and the remaining phyla accounted for 1.3% of sequences. Similarly, although 303 genera were identified, the top 25 represented 64.7% of sequences (Fig 1).

Microbial community α-diversity was significantly different between EB and BAL samples within both specimen groups at the