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The Microbiome of the Lung

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Abstract

Investigation of the lung microbiome is a relatively new field. Although the lungs were classically believed to be sterile, recently published investigations have identified microbial communities in the lungs of healthy humans. At the present time, there are significant methodologic and technical hurdles that must be addressed in ongoing investigations, including distinguishing the microbiota of the upper and lower respiratory tracts. However, characterization of the lung microbiome is likely to provide important pathogenic insights into cystic fibrosis, respiratory disease of the newborn, chronic obstructive pulmonary disease, and asthma. In addition to characterization of the lung microbiome, the microbiota of the gastrointestinal tract have profound influence on development and maintenance of lung immunity and inflammation. Further study of gastrointestinal-respiratory interactions are likely to yield important insights into the pathogenesis of pulmonary diseases, including asthma. As this field advances over the next several years, we anticipate that studies utilizing larger cohorts, multi-center designs, and longitudinal sampling will add to our knowledge and understanding of the lung microbiome.

Introduction

Investigation of the lung microbiome is a relatively new field, and may lead to new ways of thinking about respiratory disease (1). The lungs of healthy humans were previously believed to be sterile, based on results of classical, culture-based studies (2, 3). In fact, the National Institutes of Health's initial Human Microbiome Project did not include the lung as a site of investigation (4). However, recent culture-independent methods demonstrate that the lungs of healthy never-smokers are inhabited by communities of bacteria that are very few in number but composed of diverse types of bacteria (5, 6). Understanding the microbiome of the lung in normal subjects is an essential step in advancing this field. These investigations will inform studies performed in individuals with disease states classically

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considered to be infectious in origin, including cystic fibrosis (7), respiratory disease of the newborn (8), exacerbations of chronic obstructive pulmonary disease (COPD) and asthma (6, 9), and lung transplantation (10). While there are no published data to date, it is likely that changes in the microbiome could influence other clinical outcomes, such as complications occurring after lung cancer surgery (11). In this assessment, we focus on recently published work examining bacterial communities in the lung, which comprises most of the published evidence to date. Because viral (12) and fungal (13) organisms establish their own communities in the human lung, these areas require further investigation, including their interactions with bacterial communities.

Characterization of the Normal Human Lung Microbiome

Although study of the normal human lung microbiome is still in its early stages, the bulk of published evidence demonstrates that pylogenetically diverse microbial communities in the lungs of healthy humans can be detected using high throughput sequencing (6, 9, 14). While results from published studies differ, Proteobacteria, Firmicutes, and Bacteroidetes are most commonly identified at the phylum level. At the genus level, Pseudomonas, Streptococcus, Prevotella, Fusobacteria and Veillonella predominate, with lesser contributions from potential pathogens including Haemophilus and Neisseria.

Geography needs to be considered in interpretation of microbiota data. Most series published to date have examined specimens obtained from individuals in a single center, but it is reasonable to anticipate that communities would differ based on climate, environmental microbiota, exposure to other individuals, and even household pets. In one of the few studies to evaluate geographical comparisons, sputum samples from 19 cystic fibrosis patients at a single center in the United Kingdom were compared with samples from 19 patients at a single United States center (15). Differences in species were apparent between the two groups, and cluster analysis showed significant geographical differences in community composition, abundance, and diversity. A recent comparison of communities in house dust determined that bacterial diversity, evenness, and richness were increased in dust obtained from dog-owning households, and in a subset of cat-owning households, compared to dust obtained from households without pets (16).

While this scientific field is one of active development, standardized methodologic or statistical techniques have not been established, complicating comparisons among studies. Many of the published studies to date rely on small sample sizes. An additional limitation is reliance on single-sample analysis from normal volunteers or patients, but longitudinal analysis is clearly needed. Methodologic approaches also vary widely (17). Some investigations have used high throughput sequencing, while others use phylogenetic microarray analysis, terminal restriction fragment length polymorphism or amplicon length heterogeneity-polymerase chain reaction (18, 19). Importantly, each of these methodologies yields different types of information (20). There is also disparity in the selection of variable regions of the 16S rRNA gene for sequencing.

A major methodologic issue complicating investigation in this field is the possibility that lung microbiome data represent partial or complete carry-over from the naso- and/or oropharynx. It should be noted that micro- and macro-aspiration of gastric secretions occurs frequently, including patients who are undergoing conscious sedation for bronchoscopy, and in patients receiving mechanical ventilation through an endotracheal tube. While an early study, which examined endotracheal aspirates from 3 individuals who had been intubated briefly for elective surgery yielded no 16S rRNA product (21), most subsequent studies have documented the presence of microbial communities. In order to sample the lung in nonintubated individuals, invasive sampling is needed. Most investigations have utilized

bronchoalveolar lavage to sample relatively large areas of the lung. Others have utilized protected specimen brushes to sample the bronchial mucosa or the lung parenchyma. Additionally, recent work indicates that the microbiota of individual lungs differ significantly depending upon the location of sampling (6).

Most investigations moving forward perform bronchoscopy in a conventional manner, with appropriate sampling of the nose and/or mouth for comparison. However, a recent investigation in a small number of volunteers suggested that the lung may not contain a distinct microbiome and may reflect only the upper airway microbiota (5). These investigators used a two-endoscope method, in which one scope was used to perform anesthesia and a second scope was used to collect the specimen. Comparison or upper and airway sequencing date in 6 healthy individuals led the investigators to conclude that lower respiratory tract sampling yields sequence data indicative of low-level contamination with upper respiratory microbiota. Conversely, a comparison of expectorated sputum and oral washes from 19 cystic fibrosis patients demonstrated some overlap between the two specimens, but sufficiently distinct bacterial communities existed for the investigators to conclude that the sputa were not significantly contaminated by oral organisms (22). One explanation for these apparently discrepant results may be the much higher organism burden in the lungs of cystic fibrosis patients, with the upper respiratory microbiota as minor components. In normal individuals, however, it is quite possible that communities low in biomass could have significant effects on the host.

Taken together, these clinical and methodologic issues are significant hurdles that must be overcome to characterize the microbiome of the normal human lung. One reasonable way to approach this characterization is to examine data from larger, well-characterized cohorts that are sufficiently diverse clinically and geographically. One such project, currently underway, is the Lung HIV Microbiome Project funded by the National Heart Lung and Blood Institute (1). Examination of bronchoalveolar lavages and other respiratory samples from this cohort, obtained from 6 data collecting sites and analyzed by a data coordinating center, should yield data with adequate statistical power to address these questions. While the existence of stable bacterial communities in the normal lung remains a topic of controversy, it will be of critical importance to compare these data to those obtained from patients with specific diseases.

The Lung Microbiome in Disease States

Cystic fibrosis

Chronic airway infection and inflammation cause progressive lung disease in patients with cystic fibrosis, and are the leading causes of mortality in this disease (7). Traditionally, infection with known pathogens, including *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Burkholderia cepacia,* have received most attention. In addition to these organisms, however, multiple investigations have now documented the complex microbial ecology of the lung in these patients. For example, one recent study of 4 sputa from cystic fibrosis patients determined that more than 60 bacterial genera could be identified using a combination of cloning, culturing, and pyrosequencing (23). The roles of bacterial communities in the pathogenesis of disease are of increasing scientific interest, but caution must be exercised in implicating members of these communities as clinically significant pathogens (24). A study of 6 stable patients with cystic fibrosis demonstrated that, as expected, non-viable bacteria contributed significantly to profiles obtained from sputum samples (25). However, use of propidium monoazide photo-induced crosslinking can be used to exclude dead bacteria from biasing results, allowing amplification of DNA from viable bacteria. It must be noted that bacterial viability is not required to induce or to

propagate inflammatory responses, and so identification of non-viable bacteria may be clinically relevant.

A unique problem in these investigations is to accurately characterize and measure the microbiota during periods in which overwhelming infections with pathogenic bacteria are present (26). Furthermore, community pathogenicity may not be constant, and may depend on a variety of microbial and host factors (27). A comparison of culture and cloned 16S rRNA sequences from 25 cystic fibrosis patients revealed numerous discrepancies (28). While cultures revealed isolates containing 13 species known to be pathogens, sequencing data yielded 53 species (including 16 species of anaerobes). These disparities may be partly rectified by use of anaerobic culture techniques, but current culture techniques may be insufficient for organisms of low abundance, or organisms that require microbe-microbe interactions for optimal growth. Use of anaerobic techniques for bacterial culture, followed by 16S rRNA sequencing for identification, identified 12 species of Prevotella in sputa from 16 cystic fibrosis patients (29). In fact, combining culture-dependent and culture-independent approaches may increase the sensitivity of either approach (30). Using sputa from 6 patients with cystic fibrosis, investigators compared molecular profiling (terminal restriction fragment length polymorphisms and titanium sequencing) to culture techniques that included a wide variety of growth conditions and extended incubation times. Overall, 42 of 48 families detected by sequencing were cultivated. While these culture techniques are not practical for clinical practice, the most important aspects of these findings is that they demonstrate the viability of organisms identified by sequencing.

It may be useful to consider these populations separately, as “core” and “satellite” taxa. In an analysis of sputa from 14 clinically stable cystic fibrosis patients, investigators partitioned communities into “core” (dominated by *Pseudomonas aeruginosa*) and 67 other “satellite” taxa (other *Pseudomonas, Streptococcus, Neisseria, Catonella, Porphyromonas, Prevotella, Veillonella*). These distinctions may have important clinical implications, since recent antibiotic treatment and cystic fibrosis transmembrane conductance regulator (CFTR) genotype correlated with composition of the core group, but not the satellite group. Additionally, the microbiota from patients with worse lung function demonstrated decreased richness. Comparable data were obtained in a study of sputa and deep throat swabs of age-stratified cystic fibrosis patients (age range 9 months to 72 years) (31). Older patients with worse lung function demonstrated communities that were significantly less rich, less even, and had lower bacterial diversity compared with younger patients. Additionally, these investigators determined that bacterial diversity and richness decreased with age in patients heterozygous for the ΔF508 mutations to a greater extent than in patients homozygous for the ΔF508 mutation or with non-ΔF508 mutations.

In one of the few studies of viral diversity, sputa from 5 cystic fibrosis patients and 5 normal volunteers were compared (32), and viral diversity in both groups was low. The majority of viral diversity was uncharacterized, but eukaryotic viral communities in the cystic fibrosis patients were dominated by human herpes viruses and retroviruses. Importantly, functional metagenomics demonstrated that all viromes from normal volunteers were similar, but the viromes from cystic fibrosis patients were enriched in amino acid metabolism. The authors concluded that the cystic fibrosis airway environment can lead to shifts in metabolic profiles.

Bronchopulmonary dysplasia

Characterization of microbial communities in the lungs of preterm infants has been conducted to try to establish a link with the development of bronchopulmonary dysplasia. Endotracheal aspirates from 8 preterm infants at risk for bronchopulmonary dysplasia revealed a range of known pathogens including *Staphylococcus aureus*, *Pseudomonas*

aeruginosa, and *Streptococcus spp.* (33). A subsequent study examined gastric and lung fluid samples from 162 newborn infants, 35 of whom developed chronic lung disease of prematurity (8). The presence of 16S rRNA genes and *Ureaplasma spp.* was significantly associated with development of chronic lung disease. These data suggest that early establishment of microbial communities, likely leading to infection, may play a role in the development of this disease.

Chronic obstructive pulmonary disease

COPD is characterized by the presence of airflow obstruction due to chronic bronchitis or emphysema. Exposure to tobacco smoke accounts for 80 to 90 percent of the risk of developing COPD in humans. Pathologic changes seen in both the bronchioles (enlargement of bronchial mucus glands, airway smooth muscle hypertrophy and mononuclear cell infiltration) and lung parenchyma (destruction of alveolar walls) are thought to contribute to the physiologic derangement of airflow obstruction (34). Airway hyperresponsiveness is seen early in the disease and appears to identify a population at risk for cigarette-induced lung damage (35, 36). The mechanisms by which tobacco smoke lead to these changes is incompletely understood. Clearly, host factors play a role, since only a fraction of all smokers develop emphysema.

The relationship between the microbiome and COPD is currently being investigated. A recent study of endotracheal aspirate specimens obtained from 8 patients receiving mechanical ventilation demonstrated a large diversity of organisms despite antimicrobial therapy (37). Additionally, richness of the community composition decreased with longer duration of intubation. These investigators had previously demonstrated that, in 7 intubated patients colonized with *Pseudomonas aeruginosa*, bacterial diversity decreased during treatment with antibiotics (21). *P. aeruginosa* became the dominant species in these patients, and the investigators suggested that loss of bacterial diversity under selective pressure from antibiotics may be associated with the development of pneumonia. It is worth noting that microbial communities are present in the biofilms that line endotracheal tubes, and so studies of intubated and mechanically ventilated patients must consider the contributions of biofilms to data obtained from the distal lung (38). The applicability of these data to non-intubated patients, to patients without pneumonia and to patients not receiving broad-spectrum antibacterials, remains to be determined.

As discussed above for asthma, a recent study of non-intubated individuals determined that Haemophilus species were more prevalent in bronchoalveolar lavages from COPD patients than from normal controls, while Bacteroidetes were more prevalent in controls (9). A recently published study contained a preliminary analysis of the lung microbiome stratified by smoking status and pulmonary function in COPD (6). Comparison of bronchoalveolar lavage specimens from 4 COPD patients, 7 smokers with normal spirometry, and 3 never-smokers revealed bacterial 16S sequences in all subjects, but no differences among the groups. While specimens from moderate and severe COPD patients demonstrated limited community diversity, this finding was also noted in about a third of the healthy subjects. A unique aspect of this study was examination of lungs removed from 6 patients undergoing lung transplantation, showing that there were differences in bacterial communities within the same lung. Thus geographic variation among the micro-environments of individual lungs warrants further study.

Asthma

The relationship between respiratory microbiota and asthma has been suggested for many years. The incidence of allergic airway disease in industrialized countries has increased significantly in the past three decades (39). During this period of time, the rates of asthma

did not change in developing countries, suggesting that environmental challenges are a major factor in the development of asthma. One intriguing hypothesis relating asthma to the microbiome is that perturbations in gastrointestinal microbiota composition, due to antibiotic use and poor diet in westernized areas, have disrupted mechanisms involved in the development of immunological tolerance (40). Data supporting this "microbiota hypothesis" include the correlation between asthma/allergies and antibiotic use in industrialized countries (41–45) and the correlation between altered fecal microbiota and atopic disease (46–52).

Although bacterial infections may be triggers of asthma exacerbations, recent work has focused on the microbiota and its regulation in the airways of asthmatics, in attempts to establish pathogenic links (53). A study of 11 patients with asthma, 5 patients with COPD, and 8 control subjects compared nasal swabs, oropharyngeal swabs, and bronchoscopically obtained cytology brushings (9). Pathogenic Proteobacteria, particularly *Haemophilus spp.* were more frequent in the bronchi of asthmatics or COPD patients than controls, whereas Bacteroidetes, particularly *Prevotella spp.*, were more common in controls. Comparison of common phyla and genera differed among the nose, oropharynx, and lung for these subjects, but the clustering relationships as related to disease processes were less clear. A subsequent data analysis was performed on bronchoalveolar lavages from asthmatic and normal children, with similar results.

A study of 65 severe asthmatics under poor disease control, compared with 10 normal controls, examined results from bronchial brushings obtained during bronchoscopy with a protected brush (14). Both 16S rRNA amplicon concentrations (a proxy for bacterial burden) and bacterial diversity were significantly higher in asthmatics compared with control subjects. Of approximately 100 taxa that were associated with increased bronchial hyperresponsiveness, most belonged to the Proteobacteria. Although analysis of upper airway samples was not performed, a database query showed that nearly 90% of the phylotypes were note referenced to the oral cavity.

Influence of the Gastrointestinal Microbiome on Lung Immunity

Allergies and Altered Microbiota

Human investigations aimed at dissecting the relationship between the gut microbiome and lung immunity are limited due to experimental and ethical considerations. While important associational relationships are being established, direct hypothesis testing is considerably more difficult. Animal models are providing novel information to test the hypothesis that gut microbiota influence lung immunity. A murine model has been developed that uses the broad-spectrum antibiotic cefoperazone, in conjunction with low-level *Candida albicans* gastrointestinal tract colonization, to test the impact of gastrointestinal tract microbiota perturbation on the generation of allergic airway diseases (54, 55). The need for systemic allergen priming (for example, ovalbumin plus alum) in the development of allergen-induced airway inflammation was eliminated in this model. In the absence of systemic priming with ovalbumin, only mice with altered gastrointestinal tract microbiota developed airway allergic responses to intranasally administered ovalbumin. These lungs of these mice demonstrated significant increases in the number of eosinophils in the lungs, increased serum IgE levels, and increased interleukin-5 and interleukin-13 production. The eosinophilic nature of the inflammatory response was also evident in histological analysis of the lungs. In the absence of microbiota disruption, the response in the airways consisted of few eosinophils and low-level production of interleukin-5, interleukin-13 and serum IgE. The most striking change following microbiota disruption was in the development of a widespread goblet cell metaplasia. Allergic responses were also induced by intranasally-delivered *Aspergillus fumigatus* conidia, using this approach to perturb the microbiota in

either Balb/c or C57BL/6 mice but not CD4+ T cell-deficient or interleukin-13 knockout mice. Thus, animal models can be used to demonstrate that perturbation of the normal microbiota in inbred mice can promote the development of allergic airway disease following allergen challenge (54, 55).

Mucosal Tolerance and Regulatory T-Cells

A number of studies have demonstrated that fluids, microparticulates and microbes delivered intranasally will largely end up being swallowed, regardless of the volume used for delivery (56–58). Reducing the volume can prevent delivery to the lungs, but the majority of the inoculum can be detected in the gastrointestinal tract within 15 minutes (57, 58). There is increasing evidence that the gastrointestinal mucosa, the predominant site of microbiota host-interaction, can play a role in the development of immune responses at distal mucosal sites. The mucociliary architecture of the nasopharyngeal cavity and upper airways naturally sweeps all inhaled micro-particulates that stick to the mucus lining into the gastrointestinal tract. Shortly after intranasal inoculation or aerosol delivery, fluids, particles, and microbes introduced into the nasal cavity are largely found in the gastrointestinal tract (56–58). In mice, intranasal inoculation of a volume as small as 2.5 μl still largely ends up in the gastrointestinal tract (56). Thus, inhaled micro-particulates and aerosols (which comprise the vast majority of aeroallergens) are also swallowed. Using an animal model of allergic airway disease, investigators reported that two days after intranasal administration of antigenic peptide, corresponding antigen-specific CD4+ T cell division had not only occurred in the lymph nodes draining the lungs and nasopharyngeal cavity, but also in the mesenteric lymph nodes (59). No division was seen in peripheral non-draining nodes. Thus, tolerance to intranasally and orally delivered antigens likely involve overlapping, if not identical, mechanisms. Oral tolerance can block the development of IgE and allergic airway responses (60–63). In systemic models, oral tolerance is mediated by antigen-specific CD25+CD4+ T cells that produce either interleukin-10 or transforming growth factor-β (64, 65). It remains to be determined how these distal mucosal sites interact in generating mucosal immunity.

The indigenous microbiota and mucosal tolerance

The importance of the gastrointestinal tract microbiota in the generation of mucosal immune responses and mucosal tolerance was first demonstrated using germ-free mice. Early studies indicated that oral tolerance could not be induced in germ-free mice (62, 63, 66), however feeding germ-free mice normal microbiota restored oral tolerance induction and regulatory T cell induction. Microbiota-mediated toll-like receptor (TLR4) signaling is likely critical for the generation of T-cell mediated oral tolerance. Mice lacking expression of TLR4 displayed heightened IgE-mediated allergic responses upon oral food allergen administration that correlated with Th2-mediated T-cell cytokine responses (67). Furthermore, treatment of wild-type mice with a cocktail of antibiotics to reduce the microbiota resulted in mice becoming susceptible to food allergens to a similar degree as TLR4-deficient mice (67, 68). The requirement of the microbiota to provide the required signal was demonstrated in germ-free mice monocolonized with the commensal bacteria *Bifidobacterium infantis*, which reconstituted the ability of these mice to generate oral tolerance (63). More recently, it has been demonstrated that germ-free mice sensitized to ovalbumin develop exaggerated airway eosinophilia, and increased production of IgE and Th2 cytokines, compared with specific pathogen-free mice (69). Colonization of the germ-free mice with the commensal microbiota of the specific pathogen-free mice abrogated these responses. One of the major sites of mucosal tolerance induction appears to be the mesenteric lymph nodes, a site of interaction between gastrointestinal tract bacteria and mucosal immune system (70, 71). It was recently reported that the composition of intestinal microbiota regulates the Th17:Treg balance in the small intestine lamina propria (72). Altogether, these studies support the concept that the

indigenous microbiota plays a critical role in regulating mucosal immunity, tolerance, and susceptibility to inflammatory diseases.

Probiotics are live microbes, that when delivered in sufficient quantity, can promote health. The most widely studied probiotics are of two genuses: *Lactobacillus* and *Bifidobacterium*. Several reports have studied the effect of strains of *Lactobacillus* in modulating allergic pulmonary inflammation (73–75). Interestingly, *Lactobacillus reuteri* and *Lactobacillus plantarum* reduced while *Lactobacillus casei* augmented allergic airway inflammation. Interestingly, a number of strains of *Lactobacillus casei* have shown positive effects for treating bacterial and viral pneumonia in mice (76–78).

A number of orally delivered microbial products, can modulate experimental allergic airway disease. For example, pre-treatment with heat-killed *Mycobacterium vaccae*, a ubiquitous saprophytic mycobacterium, either by subcutaneous or by intragastric administration can inhibit the development of allergic airway disease (79–81). Moreover, treatment prevented Th2 sensitization through stimulation of inhibitory dendritic cells and Treg cells, and not through generation of a Th1 response. In another study, oral administration of oligodeoxynucleotides containing bacterial CpG motifs (TLR9 ligands) inhibited some parameters of allergic airway disease in an ovalbumin-induced asthma model (82). Respiratory exposure to the TLR4 ligand, LPS, can augment allergic responses in a rather complex pattern that is at least in part dose dependent (83–85). The ability of certain microbial products to induce immunologic tolerance to allergens has lead to clinical testing for the treatment of allergic rhinitis and asthma (86).

Summary

Study of the lung microbiome is a relatively new field, and this review summarizes recently published literature that demonstrates the presence of bacteria in the lower respiratory tract by culture-independent methodologies. Although the lungs were classically believed to be sterile, recently published investigations have identified microbial communities in the lungs of healthy humans. There are significant methodologic and technical hurdles that must be addressed in moving this field forward, including distinguishing the microbiota of the upper and lower respiratory tracts. Because the scientific community engaged in this work has not reached consensus on methods for sampling, analysis, or data presentation, comparisons among studies can be problematic. However, characterization of the lung microbiome is likely to provide important pathogenic insights into cystic fibrosis, respiratory disease of the newborn, chronic obstructive pulmonary disease, asthma, and other respiratory illnesses. Looking beyond characterization of lung microbial communities, it is now apparent that the microbiota of the gastrointestinal tract have profound influence on development and maintenance of lung immunity and inflammation. Further study of gastrointestinal-respiratory interactions are likely to yield important insights into the pathogenesis of pulmonary diseases, including asthma. As this field advances over the next several years, we anticipate that studies utilizing larger cohorts, multi-center designs, and longitudinal sampling will add to our knowledge and understanding of the lung microbiome.

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Abbreviations

COPD	chronic obstructive pulmonary disease
CFTR	cystic fibrosis transmembrane conductance regulator
TLR	toll-like receptor

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