

Chapter 7

The Human Lung Microbiome

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The Human Lung

Lung Microbiome Overview

The human LOWER respiratory tract is considered sterile in normal healthy individuals (Flanagan et al., 2007; Speert, 2006) despite the fact that every day we breathe in multiple microorganisms present in the air and aspirate thousands of organisms from the mouth and nasopharynx. This apparent sterility is maintained by numerous interrelated components of the lung physical structures such as the mucociliary elevator and components of the innate and adaptive immune systems (discussed below) (reviewed in (Diamond et al., 2000; Gerritsen, 2000)). However, it is possible that the observed sterility might be a result of the laboratory practices applied to study the flora of the lungs. Historically, researchers faced with a set of diseases characterized by a changing and largely cryptic lung microbiome have lacked tools to study lung ecology as a whole and have concentrated on familiar, cultivatable candidate pathogens. With the availability of new technologies for cultivation-independent analysis of microbial populations, it is now possible to follow individuals by sampling their lung microbiome sequentially during episodes of disease and recovery in order to identify associations between the lung microbiome and health and disease.

Any respired contaminating particles or pathogens that evade the lung's physical and immune barriers are usually eliminated by dendritic cells and alveolar macrophages that deliver them into local draining lymph nodes. Macrophages kill invading microorganisms while *en route* to the draining lymph nodes, and in some cases at the nodes themselves (Bozza et al., 2002; Kirby et al., 2009). Thus, it is not unusual to isolate viable bacteria or fungi from “normal” lung tissue (Lass-Flörl et al., 1999), and the term “sterile” should be applied with caution. It is perhaps

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more accurate to say that there is no resident flora that permanently colonizes normal lungs.

Even normal healthy lungs are not microbe free all the time. Lower airway infections by bacteria, viruses, or fungi are among the most prevalent causes of transmissible disease in humans, with two to three million community-acquired (non-hospital-acquired) cases per year in the United States (Segreti et al., 2005). In 2006, the number of deaths attributed to pneumonia (bacterial and viral) and influenza in the United States was 60,000 (Gao et al., 2008) www.cdc.gov/nchs/fastats/deaths.htm. In 2009, nearly 9.3 million new cases of tuberculosis were reported around the world (<http://www.who.int/mediacentre/factsheets/fs104/en/index.html>). With proper treatment, the lungs of individuals with these infectious diseases will revert to their normal “sterile” state.

Little is known about the composition of the microbial population of the upper and lower airways in health or disease. It is likely, given the multiple microorganisms already implicated in chronic lung diseases such as chronic obstructive pulmonary disease (COPD), that there are other undetected organisms and that there are complex relationships between multiple pathogens involved that are not currently understood. A few studies have examined microbial species in limited numbers of normal subjects and patients with various respiratory disorders. One study using 16S rDNA clone libraries and microarrays did not detect any bacteria in the lungs of patients without respiratory disease who were briefly intubated for surgery (Flanagan et al., 2007). The same study reported that all patients intubated for longer periods had detectable 16S rDNA and that the bacterial diversity present decreased during antibiotic usage. Another study used 16S rDNA amplification to identify bacterial species in 16 patients with ventilator-associated pneumonia (Bahrani-Mougeot et al., 2007). This study identified bacterial pathogens not seen using conventional culture techniques, especially anaerobes, and found that oral bacteria could be detected in the lung. In one study, sputum samples from 25 cystic fibrosis (CF) patients were analyzed using 16S gene profiling and the authors identified an average of 7.2 species present per subject (Bittar et al., 2008). Viruses have also been examined in nasal lavages in both asthmatic and normal subjects with cold symptoms using the Virochip microarray (Kistler et al., 2007). The microarray technology identified more viruses than conventional culture methods and had excellent sensitivity and specificity compared with pathogen-specific polymerase chain reaction (PCR). An unexpected diversity of human coronavirus and rhinovirus strains was discovered in the subjects.

So if the lung is generally sterile, why do some individuals become chronically colonized? What organisms colonize the lungs? Those with physically compromised airways or immune system deficiencies are subject to chronic microbial colonization of their airways and to high-frequency episodes of viral, bacterial, or fungal lower respiratory infections. Perhaps in no other body site is the direct relationship between disease and microbiome more explicit than in the lungs where there is a distinct and obvious microbial difference between normal and diseased individuals.

Anatomical and Immunological Setting

The lower respiratory tract, composed of the trachea and lungs, is quite different in structure and function from the upper respiratory tract, which is highly colonized by microorganisms. The lungs themselves are divided into different sections according to their function and structures: the bronchi, bronchioles, and alveoli. Bronchi and bronchioles are primarily conductive airways surrounded by thick cartilage that allow easy airflow into the parenchyma (or alveolar tissue) of the lungs, where gas exchange occurs. Conductive airways are covered in ciliated epithelium interspersed with different types of secretory cells that release mucins, immunomodulatory proteins, surfactants, and proteases. Together, the physical and chemical barriers protect against physical and biological damage by establishing a mucociliary elevator, which brings about an upward transport of a mucus stream for the lungs (Fraser, 2005). The secretory cells decrease in proportion from 20 to 30% in the trachea to less than 1% in the distal and alveolar parts of the lungs. In addition to the physicochemical protection provided by cilia and mucus, the epithelium is also protected by several immune cells, including dendritic, Langerhans, T lymphocytes, and mast cells, that respond to inhaled antigens establishing a robust immunity (Fraser, 2005). It is thought that in conjunction with the physical barriers provided by the nose and upper respiratory mucosa, these defenses are enough to maintain sterility in the lower respiratory tract.

The vast majority of the lung surface epithelium, however, is alveolar. It is estimated that 87% of the total volume of the lungs is alveolar, with only 6% of this being tissue and the remainder gas (Stone et al., 1992). The primary role of this tissue is to carry out gas exchange. The epithelium is mostly a continuous single layer of cells overlying a thin interstitium, which contains numerous capillaries that supply ample blood for gas exchange (Fraser, 2005; Stone et al., 1992). Unlike the epithelium in the conducting airways, the respiratory epithelium is not ciliated or protected by mucus. Instead, it is covered by surfactant proteins that maintain the surface tension for efficient gas exchange. The lack of mucus or secretory cells is compensated by the presence of alveolar macrophages, mast cells, lymphocytes, dendritic cells, and other monocyte-like cells that protect the epithelium from potential pathogens and help maintain sterility.

Diseases of the Lower Respiratory Tract and Their Impact on the Lung Microbiome

Recent World Health Organisation (WHO) figures rank lower respiratory diseases second in an assessment of the burden of disease worldwide (<http://www.who.int/respiratory/en/>). In 2006, 124, 500 people died in the US due to chronic lower respiratory disease (www.cdc.gov/nchs/fastats/deaths.htm). Chronic respiratory diseases include: asthma, COPD, CF, and bronchiectasis. These diseases generally lead to impaired clearance and function of the mucociliary elevator and/or the immune

protection of the lung. In addition, immune deficiency such as that caused by the human immunodeficiency virus (HIV) also disrupts the typical immune homeostasis in the lungs. Without the normal protective barriers, the lungs fall victim to persistent and severe colonization that can ultimately lead to death, particularly for CF patients. As discussed below, the lung microbiome in each of these diseases is very different from normal individuals. The data discussed in the following sections demonstrate a clear link between microbial colonization and severity of disease symptoms. It is unclear, however, what exact role these different microbial populations play in initiating and enhancing the progress of such chronic respiratory diseases. Lastly, in some cases, the data hint that some population structures might actually be protective against further decline, but much more research needs to be conducted in this area to make a definitive claim.

This chapter discusses the methods for sampling and characterizing the microbiome of the lungs. In addition, it reviews the current status of our understanding of the lung microbiome in asthma, idiopathic bronchiectasis, CF, COPD, and during immune deficiency due to HIV infection.

Microbiome Characterization

Human Lung Sampling Methods

Several procedures have been developed for sampling the microbial population of the human lung niche. In order of increasing invasiveness they are sputum induction, bronchoalveolar lavage (BAL), bronchial brushing, endobronchial biopsy, and transbronchial biopsy. Sputum induction by inhalation of hypertonic saline is a non-invasive method to obtain samples from the lower respiratory tract for cell and microbial analysis (Bickerman et al., 1958). The quality of samples varies and can be scored on the volume of the obtained sputum plugs and the level of salivary contamination as measured by squamous cells observed by microscopy.

BAL is a procedure in which a bronchoscope is passed through the mouth or nose into the lungs and saline is instilled into a segment of the lung and then recollected for examination (Henderson, 1994; Reynolds and Chretien, 1984). BAL is most commonly used to diagnose infections in both immunocompetent and immunosuppressed patients. BAL is the most common procedure for sampling the lower respiratory system microbial colonization/infection status, to sample the components of the epithelial lining fluid, and to determine the protein composition of the airways. It is often used in evaluating the patient's lung immunological status by sampling cells and pathogen levels. BAL is an invasive procedure and thus is less ideal for research purposes.

Bronchial brushing provides access to cells and microbes that are adherent to the luminal surfaces of the lower airways. In this procedure, a flexible fiber optic bronchoscope is used for brushing a targeted lesion or site (Fennessy, 1967; Zavala et al., 1973), where induced sputum and BAL procedures will allow sampling of cells and microbes that can be washed from the lumen surface, brushing will recover adherent

cells (e.g., bronchial epithelial cells) and microbes. Recently, brushing techniques have been developed to sample distal lung (i.e., small airway) epithelial cells and associated microbes (Ammous et al., 2008). This technique will enable investigators to further study the microbiome in lung diseases such as COPD.

Endobronchial biopsy involves using the fiber optic bronchoscope to identify appropriate target sites in the lung and obtain large airway tissue samples using inserted alligator forceps, cup forceps, or curette passed through the endoscope's central channel. This procedure poses a higher risk than BAL but allows sampling the invasive microbes within the airway tissue (Scott et al., 1991; Trulock et al., 1992).

Transbronchial biopsy, the most invasive of these sampling procedures, is routinely performed for clinical care and allows clinicians and researchers to obtain distal (small) airway tissues as well as alveolar tissues. This procedure has been safely done by several research groups (Balzar et al., 2005) and will likely further our understanding of the microbiome in human distal lung tissues, but carries a significant risk of complications.

High-Throughput Sequencing of Bacterial Ribosomal RNA Subunits

Standard microbiological and virologic methods detect only a small proportion of the bacteria and viruses present in various body sites because the great majority of these organisms are uncharacterized or uncultivable. To understand the real diversity, culture-independent methods, such as sequencing, are thus a necessity. Sequenced-based identification of microbial species is facilitated by decreased costs of sequencing, and the availability of next-generation sequencing technologies, further enhances the capacity to generate large amounts of data.

For the identification of bacterial species within an environment, the amplification of 16S rRNA genes (or 16S rDNA) using universal primers are useful for diversity characterization because this genetic locus is present in all bacterial species (Relman et al., 1991). The nine hypervariable regions of the 16S rDNA can be used for bacterial species identification (Chakravorty et al., 2007; Rokas et al., 2007) with some regions having better discriminatory value than others. The sequencing and phylogenetic analysis of bacterial 16S rRNA derived from microbiome samples has been the primary method used to investigate bacterial diversity in the human body (Bik et al., 2006; Dekio et al., 2005; Eckburg et al., 2005; Gao et al., 2007; Hugenholtz et al., 1998; Hyman et al., 2005; Zhang et al., 2006). These studies have revealed a far higher level of diversity than conventional culture techniques (Aly et al., 1976; Bik et al., 2006; Dekio et al., 2005; Kazor et al., 2003; Korting et al., 1988; Kroes et al., 1999; Paster et al., 2001). These studies revealed that the majority of bacterial sequences correspond to uncultivated species and novel organisms. There was significant intersubject variability and variability between stool and mucosal microbial populations. For example, recent studies by Blaser and colleagues at New York University have demonstrated substantial changes in the ratio of the genus *Streptococcus* to *Propionibacterium*

in skin samples from healthy persons and in normal skin of patients with psoriasis (ratio = 0.4; $n = 2,649$ clones), and from psoriatic lesion samples (ratio = 5.0; $n = 1,314$ clones; $P = 0.01$) (Gao et al., 2008).

In a lung study, bacterial diversity was analyzed in the endotracheal aspirates from seven intubated patients colonized with *Pseudomonas aeruginosa* using both sequencing from 16S rRNA clone libraries and an oligonucleotide microarray termed as the PhyloChip (Flanagan et al., 2007). Controls were subjects briefly intubated for elective surgery. Bacteria were not detected by either method in samples from the controls. Sequencing from the clone libraries detected the presence of many orally, nasally, and gastrointestinal associated bacteria including known pathogens. The Phylochip detected the same organisms and many additional bacterial groups present at low abundance. Following antibiotic therapy, the bacterial populations' diversity decreased and was dominated by a single respiratory pathogen. In six of the seven patients, the dominant species was *P. aeruginosa* in spite of targeting this organism with antibiotics to which it was reportedly sensitive. The authors hypothesize that the loss of population diversity may directly contribute to pathogenicity, persistence, and development of pneumonia.

Ribosomal RNA ITS Typing of Fungal Populations

Similarly, amplification of regions from the 18S and internal transcribed spacer (ITS) regions of the rRNA, a conserved fungal gene, allows discrimination of fungal species (Fujita et al., 2001; Makimura, 2001). A preliminary study was undertaken to examine the efficacy of a community sequencing method to identify the fungal species in BAL lung samples from 23 human subjects. The fungal ITS1-5.8S-ITS2 region was amplified and the results showed that 4 of 23 patients (17%) had fungal DNA levels that could be reproducibly detected by PCR. The detected fungi included *Aspergillus fumigatus*, *Candida tropicalis*, and *Penicillium digitatum*, among others (Denning, unpublished). These data agree with a culture-based study that showed that 63% of their sample population had evidence of pulmonary fungal colonization (Lass-Flörl et al., 1999), most commonly with *A. fumigatus* and other *Candida* species, and also Zygomycetes. The results also demonstrate that rRNA sequencing is a viable platform for characterization of fungal communities in the human body.

Viral Identification via Genome Sequencing for Population Analysis

Although there are no conserved genes that can be targeted for determination of viral diversity, whole genome shotgun of a sample enriched for viruses (such as by filtering) can lead to an effective characterization of viral communities (Angly et al., 2006). Hundreds of viral genome sequences can be completed in a single sequencing reaction run using the GS-FLX (454/Roche) sequencing platform. Using

this technology and a random priming-based method, referred to as sequence independent single primer amplification (SISPA), near-full-length genomes of RNA or DNA viruses can be sequenced. SISPA can be used to sequence known and unknown viral genomes (Djikeng et al., 2008). This viral sequencing methodology can potentially be adapted for the determination of viruses within BAL by enrichment using nuclease treatment and filtration followed by taking the extracted total RNA and DNA through the SISPA process followed by sequence comparisons to known viruses.

Metagenomics

The initial studies of small 16S rRNA described above hinted at great diversity within the human microbiome, yet it left important questions unanswered such as the identity of the nondominant community members and their biological roles. The applications of shotgun techniques to the study of the human microbiome (Kurokawa et al., 2007; Manichanh et al., 2006; Zhang et al., 2006) again highlight the extent of microbial diversity associated with the human body while revealing much more of the identity and biology of nonculturable microorganisms. As a result of reduced costs and improved sequencing technologies, it is possible to perform in-depth metagenomic surveys of the human body's microbial diversity beyond the 16S rRNA surveys. Metagenomics, a term introduced in 1998, describes the functional and sequence-based analysis of total microbial genomes from environmental samples (Handelsman et al., 1998). Metagenomics uses techniques that resemble the "Whole Genome Shotgun" approach of single genome sequencing, but it is not limited to a single species.

Human metagenomics has provided insight into the complex composition of the microbiome of these several body sites, and this information has allowed us to draw tentative conclusions about the relationship between specific microbiomes and health. The human microbiome is composed of multiple "ecological niches", including the mouth (Kroes et al., 1999; Paster et al., 2001), esophagus (Zhang et al., 2006), stomach (Bik et al., 2006), intestine (Gill et al., 2006), skin (Gao et al., 2007), and vagina (Zhou et al., 2004). Our understanding of the overlap and the degree of communication between them is rudimentary at best. Perhaps the most extensively studied has been the human gut microbiome where the interaction of the gut microflora, independently or through interaction with the genetic makeup of the host plays a role in obesity, Crohn's disease, and ulcerative colitis (Frank et al., 2007; Gophna et al., 2006; Turnbaugh et al., 2006).

The Lung Microbiome in Asthma

Asthma is a complex disease characterized by chronic inflammation in the lungs and reversible narrowing of the airways. Symptoms include dyspnea, coughing, wheezing, airway hyper-reactivity, chronic eosinophilic atopy, and mucus hyper

secretion (Busse and Lemanske, 2001). About 20 million people in the US have been diagnosed with asthma; 9 million of them are children. Asthma causes 4,000 deaths per year in the US and 11 million exacerbations. Asthma is caused by environmental and genetic factors (Martinez, 2007), with asthma attacks resulting from immune responses to inhaled allergens. The majority of asthma exacerbations are caused by viral infections (Krishnan et al., 2006). Atypical bacterial infections have also been associated with asthma exacerbations and with chronic asthma (Johnston and Martin, 2005; Martin et al., 2001). In susceptible individuals, the development of asthma has been associated with bacterial colonization in neonates (Bisgaard et al., 2007) and viral and bacterial infections (Wu and Chu, 2009). There is abundant evidence testifying to the importance of microbes to the development and maintenance of asthma. A recent publication using a bacterial gene sequencing method suggests a disordered microbiome in asthmatic airways (Hilty et al., 2010).

In the developed world there has been an increased focus on predisposing factors for asthma due to its rapidly increasing prevalence, now affecting up to a quarter of urban children (Lilly, 2005). Asthma is known to be caused by environmental and genetic factors (Martinez, 2007). These factors determine asthma severity and how easily it can be treated (Martinez, 2007). Many associations with asthma have been detected including exposure to cigarette smoke (Thomson et al., 2004), caesarean section birth relative to natural birth (Thavagnanam et al., 2008), early viral respiratory infections (Gold and Wright, 2005; Harju et al., 2006), early in life antibiotic use (Marra et al., 2006), and living in the US (Gold and Wright, 2005). One theory for the cause of the increase in asthma incidence is the hygiene hypothesis (Strachan, 1989), that the rise in prevalence of asthma is a direct consequence of the success of modern hygienic practices in preventing childhood infections. This hypothesis is supported by numerous studies that have shown that children coming from a less hygienic environment have less asthma and other allergenic diseases (Ball et al., 2000; Celedon et al., 1999; Jarvis et al., 1997). In addition, alterations in innate immune system genes have been shown to be associated with the inception and development of asthma. These genes include the toll-like receptors and other genes such as *MBL*, *MYLK*, *DEFB1*, *JUN*, *INF- α 5*, and *NOS2A* reviewed in Wu and Chu (2009).

The Role of Infections in Asthma Exacerbations

Asthma exacerbations have long been associated with viral infections (Pattimore et al., 1992). More recently, the use of reverse transcriptase PCR has greatly facilitated the identification of the exacerbation-associated virus. Studies using this tool have suggested that 75–80% of asthma exacerbations are caused by virus infections (Wark et al., 2002).

Rhinovirus (RV) infections during early childhood are associated with the development of asthma, lower respiratory tract infections, and wheezing (Jackson et al., 2008; Lemanske et al., 2005). They are also associated with hospitalization for

asthma in adults (Venarske et al., 2006). A separate study revealed that patients with allergic asthma infected with RV had increased admissions to hospitals and that dust mite allergen was the primary allergen when these patients were skin tested with a panel of aeroallergens (Green et al., 2002). Respiratory syncytial virus (RSV) in infants causes lower respiratory infection leading to pneumonia and bronchiolitis. RSV bronchiolitis is the leading cause of wheezing in infants and young children, and children infected with RSV resulting in bronchiolitis are more likely to develop wheezing and asthma later in childhood (Peebles, 2004). Similarly, the human metapneumovirus (hMPV) was first isolated from children in 2001 and has been found to be associated with asthma exacerbations in both children under 5 years of age and adults (Foulongne et al., 2006; Williams et al., 2004).

Mycoplasma pneumoniae and *Chlamydia pneumoniae* are bacteria that attach to airway epithelial cells and cause cell damage. Infections by these bacteria have been shown to be associated with asthma exacerbations (Johnston and Martin, 2005; Lieberman et al., 2003; Martin et al., 2001). Using a PCR assay, 31 of 55 patients with asthma were positive for either of these bacteria in lung tissue or BAL, suggesting that some level of colonization by these bacteria may be common in asthma patients (Martin et al., 2001). Studies in a mouse model suggest that preexisting allergic inflammation impairs the ability to upregulate TLR-2 and IL-6 in the lungs, leading to decreased clearance of *M. pneumoniae* and an increase in airway inflammation (Kraft et al., 2008).

The Role of Infections and Microbial Colonization in Asthma Development

Evidence is accumulating that infections are associated with the induction and development of asthma. First, long-term cohort studies on the development of asthma show that most childhood asthma begins in infancy. The first episode of wheezing begins before the age of 3 and is frequently associated with lower respiratory tract viral infections, usually RSV, but also RV (Gern et al., 2000; Sigurs et al., 2005). These infectious episodes and associated wheezing are strong predictors for the development of childhood asthma and atopy (Devulapalli et al., 2008; Kusel et al., 2007; Martinez et al., 1995; Singh et al., 2007). Second, many studies have associated viral infections with asthma prevalence in children (Devulapalli et al., 2008; Jackson et al., 2008; Kusel et al., 2007; Papadopoulos and Kalobatsou, 2007; Sigurs et al., 2005; Singh et al., 2007; Williams et al., 2004). Lastly, Wu et al. have provided evidence to suggest that viral infections have a causal role in asthma initiation and development where they show that viral infection during the first 4 months of age is strongly correlated with the development of asthma by age 5 (Wu et al., 2008). Only one-third of children with childhood wheezing and asthma, however, will develop persistent asthma symptoms in adulthood (Gerritsen, 2002; Taylor et al., 2005; Vonk et al., 2004). Management of the symptoms with corticosteroid therapy is effective but may not alter the asthma progression (Guilbert et al., 2006).

The role of infections in asthma induction and development will likely be shown to be mediated through the effect of these infections on the chronic inflammatory response in the airways of asthmatics. Microbial infections can generate either a Th2- or a Th1-biased response that could exacerbate or attenuate asthma, respectively. In asthmatics, a pro-inflammatory Th2 response persists even in the absence of allergens involving CD4+ Th2 cells, eosinophils, mast cells, and the Th2 cytokines IL-4, IL-5, IL-9, and IL-13 (Holgate, 2008).

Bacterial infections have been shown to contribute to asthma development. In a longitudinal prospective birth cohort study of 411 infants born to mothers with current or previous asthma, neonates colonized in the hypopharyngeal regions with *Streptococcus pneumoniae*, *Haemophilus influenzae*, or *Moraxella catarrhalis* or a combination of these organisms were found to be at increased risk for recurrent wheezing in early childhood and asthma at age 5 (Bisgaard et al., 2007). A protective role for some bacteria has been reported (Blaser et al., 2008). Several studies have found a protective effect of mycobacterial exposure on atopy and airway inflammation (Camporota et al., 2003; Shirakawa et al., 1997; Yang et al., 2002). These exposures include Bacillus Calmette-Guerin vaccination or heat-killed *Mycobacterium vaccae*. Early exposure to bacterial endotoxins may reduce future allergies or asthma (von Mutius et al., 2000), although endotoxins associated with house dust are associated with more asthma symptoms and worse lung function (Dales et al., 2006; Michel et al., 1996; Park et al., 2001).

Thus, the role of bacteria in asthma initiation and development appears to be complex. The causative interaction is likely to prove to be the interaction of bacteria and bacterial components in modulating the Th1 and Th2 innate immune system responses. The characterization of these interactions will be complicated by timing, dose, anatomical site, and duration of the bacterial exposure as well as the host genetic and environmental factors influencing the immune inflammatory response (Holt, 1996).

The Role of Fungi in Severe Asthma

It has recently been demonstrated that patients with severe asthma who are also atopic or sensitized to environmental fungi may benefit from treatment with the antifungal azole itraconazole (Denning et al., 2009). This observation has raised questions about the relationships among asthma severity, fungal sensitization, and fungal exposure. The issue is complicated by more than 1.5 million species of fungi that are thought to exist (Hawksworth and Rossman, 1997) and more than 80 species of fungi that have been associated with symptoms of airway allergy (Horner et al., 1995). For one species, *A. fumigatus*, 20 allergens are thought to participate in human airway allergies (www.allergome.org). Determining the clinical relevance of fungal allergens is confounded further by extensive cross-reactivity among fungal allergens (Cramer et al., 2009).

Fungal allergens can induce a number of different human bronchopulmonary disorders, each with a distinct immune pathogenesis. In allergic bronchopulmonary

aspergillosis (ABPA), the respiratory system is chronically colonized typically with *A. fumigatus*. Evidence now suggests that severe asthmatics without ABPA are more likely to be atopic to fungi than patients with milder disease. The diagnostic label “severe asthma with fungal sensitization (SAFS)” has recently been applied to this group (Denning et al., 2006). In these patients, the fungal sensitization is most commonly *A. fumigatus*, *Candida albicans*, and *Penicillium notatum* (O’Driscoll et al., 2009).

The association between severe asthma and fungi has been identified in numerous studies. Atopy to environmental fungi has been associated with severe asthma (O’Driscoll et al., 2005). Many population studies have shown an association between local fungal spore counts and medical emergencies due to asthma exacerbations (Atkinson et al., 2006). Furthermore, studies have shown that fungus exposure in fungal-sensitized individuals induces asthma symptoms (Malling, 1986; Matheson et al., 2005; Pulimood et al., 2007; Salo et al., 2006; Woodcock et al., 2006). Treatment of patients with SAFS with antifungal drugs has generally led to improvement of asthma symptoms (Denning et al., 2006) concurrent with improvements in several markers of atopy such as reduced IgE values, reduced eosinophils counts, and reductions in the level of dose of oral and systemic steroids required (Pasqualotto et al., 2009).

These findings lead to the considerations of the fungal composition of the lung microbiome in asthmatic individuals and indeed in normal individuals. Environmental fungi colonize the lungs of otherwise healthy people (Lass-Florl et al., 1999; Okudaira et al., 1977). These studies were dependent on cultivation-based methods for the detection and identification of these fungi. As a cultivation-independent method, gas chromatography/mass spectroscopy on exhaled breath has revealed the presence of fungus specific biomarkers in patients with CF with and without fungal colonization by *A. fumigatus* (Syhre et al., 2008). This approach was limited to analyzing for known *A. fumigatus* markers. The application of sequencing-based approaches for studying the lung microbiome will be essential for revealing the role of fungi in the lung microbiome and the role of the lung microbiome on asthma.

The Lung Microbiome in Cystic Fibrosis (CF)

Introduction

CF is the most common inherited lung disease in the world. It is a severe autosomal recessive disease with an incidence of 1:2000 at birth in populations of northwestern European origin, with a mutant gene carrier frequency of 1:23 in these populations. The genetic defect occurs in the cystic fibrosis transmembrane regulator (CFTR) protein, which acts to transport chloride across cell membranes. Patients with CF are the archetype population with chronic bronchial colonization. Symptoms include permanent bacterial colonization of the lower airways, with a formation of a biofilm, fat maldigestion, male infertility, and elevated levels of

chloride in the sweat (Knowles and Durie, 2002). The thick pulmonary system mucus in CF patients minimizes the effectiveness of the mucociliary elevator in clearing the lung of mucus-trapped microorganisms and other debris. As a consequence, microbes chronically colonize these patients' lungs and they suffer bouts of infection, requiring frequent hospital admission. Cultures reveal a wide range of bacteria, including *P. aeruginosa*, *Mycobacteria*, *A. fumigatus*, and sometimes viruses.

The Chronically Colonized CF Airways Represent a Surprising Complex and Diverse Ecosystem

The precise contributions of different microbes to patient morbidity, and the importance of inter-specific interactions remain largely unclear [reviewed in (Harrison, 2007)]. The complexity of this ecosystem is difficult to overstate. As an example of this complexity, the lungs of CF patients contain large numbers of neutrophils that migrate to this location in response to microbial colonization. These neutrophils secrete granule antimicrobial proteins called defensins that kill microbes. By analysis of CF sputum samples, the levels of extracellular defensins are sufficiently abundant that they may damage the airway epithelium (Soong et al., 1997). As another example of this inter-specific complexity, *P. aeruginosa* in CF lungs produces copious amounts of a tricyclic compound pyocyanin that kills competing microbes and eukaryotic cells. This compound was shown to specifically inactivate a human lung epithelial cell line vacuolar ATPase (Ran et al., 2003).

The Microbiome of the CF Lung Contains Microbial Diversity Not Evaluable by Standard Culture Techniques

A study of the microbiome of the lungs was conducted to explore the hypothesis that organisms not routinely identified by culture occur in the lungs of CF patient airways and may contribute to disease. To test this hypothesis, 16S rRNA sequence analysis was performed on BAL samples from 42 subjects, 28 CF patients, and 14 other disease controls (Harris et al., 2007). The findings of this analysis were that, for CF subjects, a single rRNA type was dominantly represented in the clone libraries prepared from lung microbiome genomic DNA. This was not found in the controls. Thirteen of the CF subjects' samples contained bacteria not routinely assessed by culture. Candidate pathogens were identified in four CF subjects. Candidate pathogens were also identified in the controls. This study documented the power of culture-independent molecular techniques to provide a broader view of the airway bacteria than standard clinical culture methods.

The CF viral metagenome was explored in a recent study using five CF individuals and five individuals without disease (Willner et al., 2009). In both cohorts, the overall viral diversity was low. The CF bacteriophage communities were highly similar to each other, whereas the non-CF individual had more distinct phage

communities. CF eukaryotic viral communities were dominated by a few viruses, including human herpes viruses and retroviruses.

Fastidious or Noncultivable Bacteria: An Emerging Concept in CF

The significance of fastidious or noncultivable organisms in the airway of CF patients is beginning to be explored. Application of specific culture conditions to favor the growth of anaerobes coupled with molecular identification techniques have focused attention in CF on bacteria not routinely detected by standard culture and biochemical identification techniques (Harris et al., 2007; Tunney et al., 2008; Worlitzsch et al., 2009). Direct, culture-independent detection techniques have identified much larger numbers of bacterial species in CF airways and have demonstrated the ability to identify likely pathogenic bacteria occurring during exacerbations when routine cultures are negative (Harris et al., 2007). These molecular identification and detection methods have identified bacteria with different antibiotic susceptibilities relative to conventional pathogens and will undoubtedly lead to novel antimicrobial intervention trials in CF (Worlitzsch et al., 2009). Similar methodology to detect anaerobes or noncultivable bacteria has not been applied systematically to patients with idiopathic bronchiectasis.

The Lung Microbiome in Idiopathic Bronchiectasis

Introduction

Bronchiectasis is characterized by chronic dilation and inflammation of the conducting airways associated with recurring infections (Barker, 2002). It is the pathologic manifestation of several genetic disorders, including CF and primary ciliary dyskinesia (PCD). However, many patients have no identifiable causes. Idiopathic bronchiectasis is estimated to affect approximately 110,000 US adults (Weycker et al., 2005). Symptoms include cough and chronic sputum production, recurring airway infection, dyspnea, wheezing, and chest pain (Barker, 2002). Microbial infections are central to the pathogenesis and progression of disease.

Bronchiectasis Phenotype and Microbial Flora

Much of the research characterizing the composition and significance of the lower airway microbial flora has been done in CF and relatively little is known about the microbial contribution to disease pathogenesis in idiopathic bronchiectasis. However, recent observations suggest a link between the lower airway microbial flora and host disease characteristics. For example, the prevalence of idiopathic bronchiectasis associated with nontuberculous mycobacteria (NTM) appears to be

increasing (Billinger et al., 2009; Marras et al., 2007). Both familial clustering and a characteristic “tall asthenic” phenotype (scoliosis, pectus excavatum, mitral valve abnormalities) in postmenopausal women with bronchiectasis associated with NTM infection (Colombo et al., 2009; Kim et al., 2008) have been reported. It is unknown whether the age, female sex, and unique body morphotype associations are seen in idiopathic bronchiectasis unassociated with NTM. Correlating disease phenotype with microbial flora is dependent upon accurately categorizing the microbial status of the patients. For environmental organisms like NTM, it is important that this categorization include both accurate speciation and determination that the organism likely represents true infection rather than contamination or transient colonization. The American Thoracic Society and the Infectious Diseases Society of America (ATS/IDSA) microbiologic diagnostic criteria for pulmonary disease based on sputum specimens call for at least two positive sputum specimens for the same species (Kim et al., 2008).

Significance of Filamentous Fungi: A Novel Consideration in Bronchiectasis

Concomitant recovery of filamentous fungi from airway specimens is also common in bronchiectasis, but the pathophysiologic consequences are not known. A recent study in CF patients found that *A. fumigatus*, like NTM, was commonly present in older patients: 75% of patients aged 16–20 years and in 60% of patients over age 20 (Valenza et al., 2008). Amin and colleagues further noted that CF patients who were chronically infected with *A. fumigatus* (defined as two positive cultures in a given year) had significantly worse airway obstruction as evidenced by a lower forced expiratory volume in one second (FEV₁) and significantly higher risk of pulmonary exacerbations during subsequent follow-up than patients without *A. fumigatus* (Amin et al., 2009). This potential negative impact on the course of bronchiectasis and a possible benefit from antifungal treatment for chronic infection in CF have prompted initiation of a multicenter clinical trial of itraconazole in CF patients in Canada (Amin et al., 2009; Shoseyov et al., 2006). In non-CF bronchiectasis, a recent study suggested that *Aspergillus* is more common in patients infected with NTM than in those without NTM, and that it is commonly associated with fungal lung disease manifestations in NTM-infected patients (Kunst et al., 2006). However, outside the relatively small numbers of idiopathic bronchiectasis patients with allergic bronchopulmonary aspergillosis (ABPA) or chronic necrotizing aspergillosis, the pathologic significance of these fungi has not been systematically explored in large numbers of patients and very few data are available for *Aspergillus* species other than *fumigatus* or filamentous fungi other than *Aspergillus* (Kobashi et al., 2006; Kunst et al., 2006; Raju et al., 2008).

Serologic Assessment of Fungal Response

ABPA is well described in association with bronchiectasis occurring in asthmatics and patients with CF (Malde and Greenberger, 2004). The diagnostic criteria

rely on an elevated total IgE as well as elevated *A. fumigatus*-specific IgE and IgG in the setting of episodic bronchial obstruction, pulmonary infiltrates, and central bronchiectasis. Kunst and colleagues assessed the prevalence of positive serologic markers for *A. fumigatus* [IgE by radioallergosorbent test (RAST) and precipitins] among idiopathic bronchiectasis patients and found these markers to be commonly present especially in the setting of concomitant NTM disease (Kunst et al., 2006). Patients with these serologic markers more commonly had radiographic manifestations suggesting *Aspergillus*-associated disease. While other filamentous fungi such as *Scedosporium* species have been commonly recovered from the airways of both CF and non-CF bronchiectasis patients, the role these fungi play in disease pathogenesis remains controversial (Cooley et al., 2007). While specific IgE antibody RAST and precipitin assays can be prepared using allergen prepared from the isolated species and correlated with the clinical presentation of allergic bronchopulmonary mycoses, these assays have not been commonly used to characterize the clinical significance of these fungal species recovered from the lower airway (Fedorova et al., 2008; Lake et al., 1991).

The Lung Microbiome in COPD

Introduction

COPD is the fourth leading cause of death in the US (Petty, 2000) and is expected to rank third in the world by 2020 (Lopez and Murray, 1998). Despite efforts aimed at smoking cessation, little impact has been made on COPD incidence, and current treatments are ineffective in slowing progression of the disease. COPD has been defined by the Global Initiative for Chronic Obstructive Lung Disease (GOLD) as “a disease state characterized by airflow limitation that is not fully reversible”. The diagnosis of COPD can also encompass those with chronic obstructive bronchiolitis and emphysema. Tissue inflammation in COPD is characterized by a predominant neutrophil, CD8+ lymphocyte, and macrophage infiltration (Keatings et al., 1996; Lacoste et al., 1993; O’Shaughnessy et al., 1997; Saetta et al., 1998). It has been proposed that the mechanism of tissue damage involves the recruitment and activation of neutrophils, macrophages, and CD8+ T cells with concomitant upregulation of several cellular proteases and inflammatory cytokines.

Role of Infections in COPD

Although smoking is clearly the leading risk factor for COPD, not all smokers develop disease (Buist and Connett, 1993). While smoking can stimulate inflammation in the lungs, smokers with COPD have an increased inflammatory response than smokers without COPD, and inflammation can persist despite smoking cessation (Keatings et al., 1996; Lacoste et al., 1993; O’Shaughnessy et al., 1997; Saetta et al., 1998). These observations suggest that some other factor or factors contribute to development and perpetuation of the inflammatory response in COPD. Infection might be one such factor critical in triggering and perpetuating the inflammatory

response in COPD. The mechanism by which infections might act to promote COPD progression has been termed the “vicious circle” hypothesis (Sethi, 2000a; Sethi and Murphy, 2008). In this scenario, smoking causes structural remodeling that renders smokers more likely to become colonized and/or less able to clear subclinical infection. Defects in mucociliary clearance and surfactant abnormalities caused by smoking also contribute to the tendency to develop chronic infection (Finley and Ladman, 1972; Honda et al., 1996; Raju et al., 2008; Vastag et al., 1985; Verra et al., 1995). Once colonization is established, the organism or organisms recruit white blood cells to the lungs, stimulating release of inflammatory cytokines and chemokines as well as proteases. Inability to clear the inciting organism perpetuates the cycle, ultimately resulting in tissue destruction, airway thickening, and clinical COPD.

Bacteria, Viruses, and Fungi Have All Been Linked to COPD

The most commonly implicated bacteria are *H. influenzae*, *M. catarrhalis*, *S. pneumoniae*, and *P. aeruginosa* (Sethi, 2004). Viruses that seem to be important in COPD include adenovirus, influenzae viruses, rhinovirus, respiratory syncytial virus, and human metapneumovirus (Mallia et al., 2006; Martinello et al., 2006; Retamales et al., 2001; Seemungal et al., 2001). These pathogens can be found in patients with COPD in the stable state and during exacerbations (Sethi, 2004). The colonization seen in patients with COPD is likely playing a role in disease and is not just an innocent bystander. For example, as bacterial load increases, FEV₁ falls, and colonization has been associated with greater sputum purulence, increased sputum neutrophils, and increased levels of interleukin (IL)-8, tumor necrosis factor (TNF)- α , and neutrophil elastase (Obrian et al., 2007; Patel et al., 2002; Sethi, 2000b; Stockley et al., 2000). Exacerbations associated with viruses are more severe and last longer than those without a viral trigger (Papi et al., 2006; Seemungal et al., 2001). In addition, exacerbations associated with both bacteria and viruses may be more severe than those associated with single organisms (Obrian et al., 2007), suggesting the usefulness of metagenomic techniques in this disease.

Colonization with the fungus *Pneumocystis jirovecii* (Pc, formerly *Pneumocystis carinii* f. sp. *hominis*) has recently been implicated in COPD pathogenesis. This organism generally causes acute *Pneumocystis* pneumonia (PCP) in patients with immunosuppression such as those infected with HIV, but colonization with the organism occurs in both HIV⁺ and HIV⁻ individuals and may be important in COPD. Colonization with Pc is increased in HIV patients with COPD and correlates with disease severity (Morris et al., 2004b; Probst et al., 2000). Animal models also support the role of Pc colonization in COPD. Christensen and colleagues recently reported that in immunocompetent mice, exposure to cigarette smoke and Pc colonization resulted in pulmonary function deficits and airspace enlargement characteristic of emphysema (Christensen et al., 2008). In a model of Pc colonization in simian/human-immunodeficiency virus (SHIV)-infected nonhuman primates

(Norris et al., 2006), Pc-colonized animals developed airway obstruction and radiographic emphysema while animals infected with SHIV alone did not develop these changes (Shipley et al., 2010).

The Lung Microbiome and Human Immunodeficiency Virus Infections

Lung Diseases Remain a Leading Cause of Morbidity and Mortality in HIV Infection

Although pulmonary infections and neoplasms associated with HIV have decreased since the availability of highly active antiretroviral therapy (HAART) (Palella et al., 1998), some pulmonary conditions may actually be increasing in persons with HIV. Diseases such as COPD, asthma, and bronchiectasis were reported to be increased in those with HIV before the introduction of antiretroviral therapy, and a similar decrease in these conditions as seen in the opportunistic infections has not occurred after antiretroviral treatment of HIV. In fact, in a recent study of HIV⁺ patients, almost 4% of deaths were due to obstructive airway disease in 1998, a three-fold increase from the pre-HAART era (Louie et al., 2002). Before the HAART era, HIV⁺ subjects were noted to have an accelerated form of emphysema with significant emphysematous disease seen in subjects less than 40 years old (Diaz et al., 1992, 2000). Both emphysema and airflow obstruction have been reported in HIV infection. Unlike many of the acquired immunodeficiency syndrome (AIDS)-defining opportunistic infections, HIV-associated COPD may actually be more common in the current era of HIV as it is frequently reported in those without a history of AIDS-related pulmonary complications and the now aging HIV⁺ population has a longer exposure to smoking and HIV.

Role of the Microbiome in Obstructive Lung Diseases in HIV

Given the immunological defects seen with HIV, it is quite possible that HIV⁺ subjects, especially those who smoke, are more prone to develop subclinical pulmonary infections, even if successfully treated with HAART. The changes that occur in the lung microbiome have not been studied in HIV, but microbial colonization is a likely factor in the accelerated COPD seen in this population. The vicious circle hypothesis of COPD could be further worsened in HIV⁺ patients by upregulation of HIV levels in the lung stimulated by pulmonary colonization. Several studies have shown that pulmonary infections increase lung levels of HIV. Koziel and colleagues reported that HIV RNA was detected in 62% of patients with active lung disease compared to 16% of asymptomatic subjects, independent of clinical stage of HIV and serum HIV RNA levels (Koziel et al., 1999). The lung appears to be an independent compartment for HIV replication as drug mutations found in BAL differ from those

in blood (White et al., 2004). HIV in the lungs is associated with a lymphocytic alveolitis, particularly in those subjects with CD4 cell counts between 200 and 500 cells/ μ l, suggesting that the virus might act independently to stimulate pulmonary inflammation (Twigg et al., 1999). The relationship of HIV pulmonary viral levels, infections, inflammation, and COPD has not been examined.

Role of Pneumocystis Colonization in HIV-Associated COPD

Pneumocystis colonization is likely important in the pathogenesis of COPD in those with HIV as well as in the HIV⁻ population. In HIV⁺ subjects, the prevalence of colonization is high, particularly if subjects smoke, and colonization is seen even in patients with high CD4 cell counts receiving HAART (Morris et al., 2004a). Anatomic emphysema is also more common in HIV⁺ patients with Pc colonization (Morris, unpublished data). It has recently been shown that *Pneumocystis* colonization in HIV⁺ subjects is associated with worse airway obstruction and an increased likelihood of clinical diagnosis of COPD, independent of smoking history and CD4 cell count (Morris et al., 2009). In addition, the SHIV-infected nonhuman primates described above serve as a model for the development of COPD in the setting of Pc colonization and HIV-like immunodeficiency (Norris et al., 2006; Shipley et al., in press).

Conclusions

Microorganisms including bacteria, fungi, and viruses play a central role in development, exacerbation, and progress of lung diseases. Even though normal lungs do not have a permanent resident microbiome, diseased lungs are acutely infected and/or chronically colonized. Standard laboratory practices have not properly reflected the entirety of the microbiomes, either in health or in disease, and thus newer sequence-based technologies have begun to reveal the true complexity of the lung microbiomes. Much more research still needs to be conducted in order to fully understand the microbial burden of the lungs, and how this burden relates to health and disease.

It is apparent that conditions that compromise the physical and immune system barriers to lung colonization by microbes result in chronic colonization and recurrent infections. These conditions include chronic inflammation as seen in HIV, asthma, and bronchiectasis, or physical obstruction observed in CF and bronchiectasis. The lung microbiomes in each of these conditions has not been properly explored to date, which limits our ability to make definitive conclusions on how to best manage these diseases. Our understanding of the fundamental role of viruses in the initial establishment and progress of asthma underscores how little knowledge exists on the role of viral infection in other chronic respiratory diseases. In addition, the fact that some bacterial populations and/or components seem to be protective against further and severe exacerbations in asthma opens the door to

questions about the role of microorganisms in protecting against other diseases. The exact nature of this protection is not clearly understood, and great benefit would come from studies that further clarify these intriguing results. Furthermore, if some population structures aid in preventing disease progression, it is likely that other population structures may predispose episodes of acute exacerbations and progression of the underlying disease condition. A comprehensive understanding of the dynamics of these microbiome interactions would likely result in better, more efficient therapies for these and other respiratory diseases.

Our developing microbiome analysis technology coupled with our increasing awareness of the potential positive and negative impact of lung population structure on respiratory system health and disease strongly supports the initiation of aggressive projects to characterize the human lung microbiome and its influence on health and disease.

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