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The Bacterial Microbiota in Inflammatory Lung Diseases

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Abstract

Numerous lines of evidence, ranging from recent studies back to those in the 1920's, have demonstrated that the lungs are NOT bacteria-free during health. We have recently proposed that the entire respiratory tract should be considered a single ecosystem extending from the nasal and oral cavities to the alveoli, which includes gradients and niches that modulate microbiome dispersion, retention, survival and proliferation. Bacterial exposure and colonization of the lungs during health is most likely constant and transient, respectively. Host microanatomy, cell biology and innate defenses are altered during chronic lung disease, which in turn, alters the dynamics of bacterial turnover in the lungs and can lead to longer term bacterial colonization, as well as blooms of well-recognized respiratory bacterial pathogens. A few new respiratory colonizers have been identified by culture-independent methods, such as *Pseudomonas fluorescens*; however, the role of these bacteria in respiratory disease remains to be determined.

Keywords

Lung; Bacteria; Microbiota; Aerodigestive; Microaspiration; Disease

1. Introduction

The past five years has seen a revolution in our understanding of the relationship between the microbial world and the lung environment during health and disease. The paradigm was that, during health, the lungs were sterile [1]. The evidence for this claim was based on over a century of defining the presence or absence of bacteria in a site by our ability to culture them from tissue samples. The emergence of DNA-based culture-independent detection techniques has overcome the hurdle of identifying the precise culture conditions for identification of bacteria that may exist in a tissue site, an approach that can exert highly selective growth pressures for those bacteria [2–5]. Using techniques of molecular microbial

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identification, no studies published have supported the claim that the lungs are sterile; bacterial DNA is always detectable in respiratory samples [4, 6–12].

The upper compartments of the aerodigestive tract (mouth and nasal cavity) have been well documented to contain abundant bacteria. A number of earlier studies, some tracing back in the 1920s, demonstrated that microaspiration is common in healthy individuals [13–15], raising the idea that the lungs are continually exposed to bacteria from the upper airways. Most all of this work was performed with radiotracers applied to the nares of sleeping or sedated individuals. In one study, radioactive indium was used to study pharyngeal aspiration during sleep in 20 healthy subjects and 10 patients with depressed consciousness [14]. Almost half of the normal subjects and 70% of those with depressed consciousness aspirated during deep sleep. In those normal subjects who did not aspirate, they were noted to sleep poorly. In another study, a radioactive Tc tracer was deposited into the nasopharynx of 10 healthy sleeping subjects through a small catheter and standard lung scans were conducted immediately following final awakening. Microaspiration occurred commonly during sleep, was unrelated to sleep quality, and was variable within subjects that were studied on more than one occasion. Even more relevant to this review, they concluded that the "quantity aspirated is of an order of magnitude likely to contain bacterial organisms in physiologically significant quantities" [13]. Furthermore, as discussed by Quinn and Meyer in the 1920's, healthy human subjects, as well as other mammals, aspirate small amounts of liquids from the upper airways into the lower airways [15]. We have recently concluded studies that compared nasal, oral, lung and gastric microbiota within the same individual and have provided culture-independent microbiological support for the concept that microaspiration of upper airway microbiota is common in healthy individuals [16]. Thus, the lungs harbor bacteria even during health (the "lung microbiota"). As discussed below, the dynamics of immigration, elimination and growth of these microorganisms is markedly different from other sites of the aerodigestive tract.

2. Anatomy and Microenvironments of the Aerodigestive Tract During Health and Disease

We have recently proposed the concept that the entire respiratory tract should be considered a single ecosystem extending from the nasal and oral cavities to the alveoli, which includes gradients and niches that modulate microbiome dispersion, retention, survival and proliferation [3, 17]. The surface area of the lungs is approximately 30 times that of the skin, and a recent estimate suggest that it contains a larger surface than that of the intestinal tract [18, 19]. The airways and alveoli are continually exposed to the environment, with the linear distance from the nares to the alveoli being only about half a meter. Numerous studies, from many laboratories, on the lung microbiome during health and disease conceptually fit well into an ecological model based on the equilibrium model of island biogeography proposed by MacArthur and Wilson in 1963 [20]. Thus, we have proposed the "Adapted Island Model of Lung Biogeography" [3]. Using the concepts of this model, the composition of the lung microbiome can be predicted as arising from the outcome of three competing forces: immigration, elimination and the relative growth rates of its members. Using the language of this model, for a given site along the lower respiratory tract, the richness of bacterial species

in a healthy lung will be the outcome of the immigration and extinction rates of the bacteria that originate from the upper airways, largely the oral cavity. During health, the lungs do not provide a hospitable environment for bacterial growth. Thus, any changes in the lung microbiota that occurs during disease must result from a change in one of these three forces.

Microbial immigration can occur via inhalation of air (which contains $10^4 - 10^6$ bacterial cells per cubic meter even before reaching the microbe-dense upper airways [21]), microaspiration (which is ubiquitous among healthy subjects [13, 14]), and direct dispersion along mucosal surfaces from the upper airways. We have recently demonstrated that there is high shared membership of bacterial species between the lung microbiome and that of the mouth [16], which is consistent with other studies [7, 10]. The microbiota of healthy human lungs contrasts with that reported for air, suggesting that microaspiration contributes more to microbial immigration than does inhalation of airborne bacteria [13–15, 22–25].

Microbes are cleared from the respiratory tract via mucociliary clearance, cough (frequent even among healthy subjects [26]) and innate and adaptive immunity. The distal alveoli are bathed in pulmonary surfactant, which also has bacteriostatic activity against some bacterial strains, further creating selective pressure on reproducing communities [27]. We have recently shown that the prominent members of the lung microbiome during health are cell-associated, either as extracellular adherent or intracellular organisms [28]. Less well-studied are the potential local microbial growth conditions within the respiratory tract. While the core body temperature is 37 degrees, the air-exposed surfaces of the trachea and bronchi are significantly cooler, potentially expanding the permissive temperatures available to immigrating bacteria. Within a single lung, regional variation can be found in oxygen tension, pH, relative blood perfusion, relative alveolar ventilation, temperature, epithelial cell structure, deposition of inhaled particles and in the concentration and behavior of inflammatory cells [3, 29–31], all of which may have demonstrable effects on microbial growth rates and provide growth niches. Thus, there is still much to be learned about the biotic and abiotic factors that shape the lung microbiome during health and disease, but the composition of the bacterial communities found in the lungs during health are under pressures from the constant influx, constant elimination and restricting growth conditions of the pulmonary environment. Changes in these forces during disease will change the lung microbiome, thereby contributing the pathologic processes of the lung diseases itself, which has been described as a self-reinforcing cycle of lung disease [3, 17, 32].

Despite the description of the lung microbiome as if it is a single type of community in healthy individuals, there is subject-to-subject variation in their microbiomes, thereby creating a range of "healthy" microbiomes [4, 7–9, 11, 12, 33]. However, shifts outside of this healthy spectrum are very evident in lung diseases, as exemplified in our recent study of the lung microbiome in lung transplant recipients [34]. When analyzed at the phylum level for relative abundance, the most common phyla consistently observed have been *Bacteroides*, *Firmicutes* and, to a lesser degree, *Proteobacteria*. These are similar to those seen in concurrently collected samples from the oral cavity, but differ in relative abundance. Within these phyla, the most prominent genera observed in healthy subjects include *Prevotella*, *Veillonella*, and *Streptococcus*. While active cigarette smoking alters the microbial constitution of the upper airways [35], it has little effect on the lower airways [10].

To date, there have been no longitudinal analyses of serial respiratory specimens from healthy controls, so the relative stability or dynamic nature of the “normal” lung microbiome is unknown. Most of the longitudinal analysis of lung microbiota specimens to date has been performed on sputum specimens from patients with Cystic Fibrosis (CF) [36–41]. In these studies, the microbial communities in the sputum of individual patients were relatively stable over time, even despite the development of clinical exacerbations and the administration of antibiotics [39, 41]. Thus, while the spectrum of community compositions during health has initially been identified, It remains to be determined how dynamic the composition of the bacterial communities are in a single healthy lung over time.

Host microanatomy, cell biology and innate defenses are altered during chronic lung disease, which in turn, alters the dynamics of bacterial turnover in the lungs. Degenerative diseases such as emphysema and pulmonary fibrosis dramatically reduce the luminal surface area of the lungs, even by as much as 90% [42, 43]. Almost 75% of patients with advanced lung disease experience esophageal reflux and dysfunction, which increases the rate of bacterial microbial immigration by introducing an additional source of bacteria (stomach) [44, 45]. Mucociliary clearance is impaired in chronic airway diseases such as CF, bronchiectasis and chronic bronchitis, thereby decreasing microbial elimination. In addition, baseline mucus production is increased, thereby providing both nutrient-rich growth environments as well as pockets of decreased oxygen concentration and increased temperature [46, 47]. The importance of mucociliary clearance in controlling the airway microbiome was recently demonstrated in *Muc5b*^{−/−} mice [48]. *Muc5b* was required for mucociliary clearance and its loss resulted in defects in control of bacterial colonization and in clearance of apoptotic macrophages. *Muc5b*^{−/−} mice developed chronic airway infections by bacteria such as *Staphylococcus aureus*, *Streptococcus spp.*, and others. The single nucleotide polymorphism in *Muc5b* (rs35705950) is a risk factor for the development of interstitial pulmonary fibrosis and a recent study found that this polymorphism was independently associated with bacterial burden [49]. Inflammatory cell numbers in the alveolus and airway are increased in numbers and display higher levels of activation in individuals with chronic lung disease compared to that observed in healthy lungs, even in the absence of additional exacerbating stimuli [50, 51]. Many therapies for chronic lung disease, such as supplemental oxygen, corticosteroids, and antibiotics have known or predicted effects on bacterial growth conditions, as well as affecting immigration and elimination [2, 17].

Finally, during an exacerbation event in chronic respiratory diseases, all of these factors change even further. Exacerbations are periods of acute worsening of respiratory symptoms that arise abruptly, over hours to days, and generally prompt an escalation in medication therapy. During an exacerbation, hyperventilation accelerates the influx of air-borne microbes and microaspiration and markedly lowers airway temperature while increased cough accelerates microbial efflux [52, 53]. The number and activation state of inflammatory cells increases. Inflammatory mediators, catecholamines, increased temperature, glucose and free ATP have all been demonstrated to promote growth and virulence of selected respiratory bacterial isolates [47, 54–58]. Bronchoconstriction alters regional oxygen concentration and pH while acute mucus production and vascular permeability increase local nutrient supply. Production of mucus in the airways introduces further gradients of local anoxia and hyperthermia, which selectively favor the growth of

specific lung pathogens [46, 47, 59, 60]. Thus, the host factors that control bacterial immigration, elimination and growth are altered during chronic lung disease and exacerbation stimuli can further drive this process.

3. Detecting Bacteria in the Airways by Culture-Dependent and Culture-Independent Methods

Bronchoalveolar lavage (BAL) has been the most commonly utilized sampling technique in the study of the lung microbiome. A significant body of evidence now exists demonstrating that, despite a theoretical risk of contamination of BAL fluid by oropharyngeal bacteria during the procedure, BAL fluid is not significantly contaminated by oral microbiota adhering to the tip of the scope during passage into the lungs. For example, the route of bronchoscope insertion (oral or nasal) has no detectable impact on BAL microbiota despite the markedly divergent microbiota present in these body sites [16, 34, 61]. Studies by our lab and Morris, et al. demonstrated a significant difference between oral and BAL microbiota communities in healthy subjects [10, 16]. Segal and colleagues, using serial bronchoscopy, also concluded that bronchoscopy is a viable option for accurately sampling the lung microbiome [33].

A large number of studies have been published recently using culture-independent techniques to compare the BAL microbiota of subjects with lung disease to the BAL microbiota from healthy control subjects. All have found significant differences in bacterial community composition between healthy and diseased lungs [2]. This includes significant associations between BAL microbiota and numerous clinically significant parameters, including severity of airway obstruction, airway reactivity, inhaled medication exposure, disease prognosis, clinical response to therapeutic intervention, and cellular inflammation [9, 11, 33, 34, 62–64]. For some lung diseases, such as CF, bronchiectasis and chronic obstructive pulmonary disease, spontaneously expectorated and induced sputum has been employed for analysis. Various features of the bacterial communities detected in sputum have been significantly associated with patient age, disease severity, airway inflammation, antibiotic exposure and response to controlled viral exposure [41, 63, 65–67]. Thus, both BAL and sputum can provide meaningful microbiota signatures that correlate with other well-established host indices of lung health and disease.

4. Identification of Previously Unappreciated Bacteria in Lung Diseases

The adoption of culture-independent approaches to study the lung microbiome has also provided an opportunity to identify potential under-recognized bacterial respiratory colonizers/pathogens. The majority of these have been in the context of CF [68, 69]. New candidate pathogens include *Lysobacter* sp., *Rickettsiales*, *I. limosus*, *D. pneumosintes* and *D. pigrum*, among many others. Particular interest has arisen in anaerobes such as the *Prevotella* and *Veillonella* genera, the presence of which has also been confirmed via culture-based techniques [70, 71]. These two genera are also commonly found in healthy lung specimens, so the positive or negative implications of high-level colonization by these anaerobes in a CF lung remain to be determined. Asymptomatic subjects with HIV infection have unexpected colonization of the lung by *T. whipplei*, which is reduced by effective

antiretroviral therapy [72]. In other studies (non-CF), in which culture-based analyses have detected bacterial growth, culture-independent techniques have identified the concurrent presence of many other organisms not typically associated with disease [36, 73, 74]. We have recently reported that two prominent and distinct *Pseudomonas* species (*P. fluorescens* and *P. aeruginosa*) exist within the post-transplant lung microbiome, each with unique genomic and microbiologic features and widely divergent clinical associations, including presence during acute infection [34]. Thus, while large numbers of new bacterial colonizers have not been identified by culture-independent methods, a few new respiratory colonizers such as *P. fluorescens*, have been identified [75]. The role that these colonizers play as cofactors in lung disease is an active area of research.

5. The Microbiome and Exacerbations of Chronic Lung Diseases

Our conventional understanding is that exacerbations of chronic lung disease can reflect acute bacterial infections of the airways; however, recent culture-independent findings have challenged this view. Acute exacerbations are clinically and microbiologically distinct from acute bacterial infections (ABI) such as pneumonia. Despite the unambiguous role of airway inflammation in respiratory exacerbations, none of the studies to date have found a change in bacterial density or community diversity during exacerbation [41, 63, 66, 76–79]. In bacterial pneumonia, there is increased inflammation that is strongly associated with higher levels of bacterial colonization and diminished bacterial community diversity [3, 80]. Bacterial lung infections are the most common source of severe sepsis, while respiratory exacerbations rarely are the cause of severe sepsis and shock [81]. Antibiotics are usually effective in acute bacterial infections (ABI) while responses in respiratory exacerbations are inconsistent and run the spectrum from marked to minimal to absent [82–84] and *in vitro* susceptibility testing is useful for ABI but not for CF exacerbations [85–87]. Rather than an infection, per se, it seems much more likely that exacerbations are bouts of disorder and dysregulation of the microbial ecosystem of the respiratory tract, i.e. respiratory dysbiosis [17]. Table 1 lists the changes in the lung microbiome during exacerbations that differentiate them from those observed in ABI.

Exacerbations of chronic lung disease share key conceptual features with exacerbations of inflammatory bowel disease, another acute clinical worsening of a chronic inflammatory condition of a mucosal surface [88]. They are both associated with profound bacterial dysbiosis and dysregulated host inflammatory responses. Both include a disruption of the homeostatic balance between the resident organ microbiota and host immune mechanisms [89]. Neither has the hallmarks of an acute infection, in which one or few pathogenic species overtake a tissue site associated tissue injury. In cases where antibiotics are beneficial, the mechanism is not via eradication of a targeted bacterial pathogen; rather, it is believed to be via manipulation of bacterial community composition or indirect immunomodulatory effects of the antibiotics. In the case of macrolides, which have been reported to have some efficacy in both types of exacerbations, these antibiotics have both antimicrobial and immunomodulatory effects [90–93]. Thus, there may be analogous microbial mechanisms at play in acute exacerbations of chronic lung diseases and inflammatory bowel disease.

We have recently proposed a model of the cycle of respiratory dysbiosis and host inflammation that typifies a respiratory exacerbation [17]. In this model, an inflammatory trigger (e.g. viral infection, allergic exposure, etc.) initiates a host inflammatory response that acutely and profoundly alters the microbial growth conditions of the airways. Permeability of the airway wall and mucus production can introduce nutrient-dense substrates for bacterial growth [46, 59]. Free catecholamines and inflammatory cytokines (e.g. TNF- α , IL-1, IL-6, IL-8) can contribute to directly promoting the growth of select bacterial species, as has been reported for *P. aeruginosa*, *S. aureus*, *S. pneumoniae*, *B. cepacia complex*, a form of "interkingdom signaling" [54–58, 94]. Inflammatory cells are then recruited and activated, killing and clearing bacteria with highly variable effectiveness [95], creating a gradient of negative selective pressure across species. Airway mucus creates local niches of increased temperature and decreased oxygen tension, again selectively favoring growth of prominent disease-associated microbes [46, 47]. Emergence of new dominant members of the lung microbiome provoke further airway inflammation via pathogen-associated molecular pattern (PAMP) – pattern recognition receptor (PRR) interactions. This inflammation can further alter growth conditions within the airway, resulting in a self-amplifying feedback loop.

Bacterial exposure and colonization of the lungs during health is most likely constant and transient, respectively. It is only during a disease state that longer term bacterial colonization occurs. Tissue injury during an exacerbation would be predicted to arise via a combination of direct injury from newly-prominent community members, alteration in microbial behavior, and indirect effects of the dysregulated inflammatory response. This model of an imbalance in the host-microbe interaction that leads to tissue damage fits well into the three concepts of the "Damage-Response Framework of Microbial Pathogenesis" described by Casadevall and Pirofski [96] (Table 2). Thus, our model of the cycle of respiratory dysbiosis and host inflammation that typifies a respiratory exacerbation [17] predicts that physiologic homeostasis in the airways is restored only after the feedback loop of dysbiosis and inflammation is broken. Other host-lung microbiome interactions include the bloom of an opportunistic pathogen during immunodeficiency or the development of hypersensitivity diseases (e.g. hypersensitivity pneumonitis) during immunologic sensitization to non-pathogenic microorganisms that enter the lungs. Future studies will determine whether a similar, albeit more chronic, cycle of inflammation and microbiota disruption is central to the development, progression and maintenance of chronic lung inflammation and fibrosis.

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Abbreviations

CF	Cystic Fibrosis
BAL	Bronchoalveolar lavage
ABI	Acute bacterial infections

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Highlights

- Numerous lines of evidence, both recent molecular and radiotracer studies from the 1920's, have demonstrated that the lungs are NOT bacteria-free during health.
- Bacterial exposure and colonization of the lungs during health is most likely constant and transient, respectively.
- Long term bacterial colonization occurs only during a disease state.
- Large numbers of new bacterial colonizers have not been identified by culture-independent methods; however, a few new colonizers have been identified and the role of these in respiratory disease is unknown.

Table 1

Comparison between acute bacterial infections and exacerbations in lung diseases (adapted from [17])

	Acute bacterial respiratory infections	Respiratory exacerbations
Bacterial density (compared to baseline)	High	Normal
Bacterial community diversity (compared to baseline)	Low	Normal
Culture growth	Usually	Occasionally
Airway inflammation	High Proportionate to microbial burden	High Disproportionate to microbial burden
Sepsis	Common	Never/rare
Clinical benefit of antibiotics	Rapid, unambiguous	Inconsistent, subtle
Relevance of <i>in vitro</i> susceptibility	Critical to clinical response	No relation with clinical response

Table 2

Three Tenets of the "Damage-Response Framework of Microbial Pathogenesis" [96]

• Microbial pathogenesis is the outcome of an interaction between a host and a microorganism; it is attributable to neither the microorganism nor the host alone
• The pathological outcome of a host-microorganism interaction is determined by the amount of damage to host tissue and cells
• Damage to the host can result from microbial factors, the host response or both