Lungs, Microbes and the Developing Neonate

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Abstract

Microbes are ubiquitous on the human body and comprise approximately 90% of the cells and 99% of the genes of the human supra-organism. High throughput sequencing technology has permitted the development of culture-independent means to identify the microbiota that are unique to the various microenvironments of the body and probably contribute some function. Although the respiratory tract interfaces with the environment, the lungs were always thought to be a sterile environment until recently when these techniques were applied to healthy and disease states. Further, there appears to be a complex interplay between the development of the gastrointestinal and respiratory microbiota and the regulation of immune function. The contribution of this dynamic metabolic mass to respiratory disease in the newborn is unknown. This article will review emerging data from recent human and murine studies that suggest there is a microbial influence on the development of respiratory disease but it will also highlight many of the gaps that remain in understanding the function of the respiratory microbiome.

Introduction

The concept of humans as “supra-organisms,” that is, the eukaryotic human-specific cells and genome as well as the cumulative microbial particles including bacteria, viruses, and fungi, has recently emerged as investigations have identified that the interplay between these microbes and host cells influences health and disease. When considered as a whole, these microbes comprise approximately 90% of the cells and 99% of the genes of this supra-organism [1]. The availability of high throughput sequencing technology has permitted the development of culture-independent means to identify microbes that are present in various microenvironments and are likely to be contributing to some function. The Human Microbiome Project was developed to characterize these microbial communities and better understand their role in human health [2, 3]. A significant focus has been placed on the intestinal microbiome not only for ease of accessibility but also for the complex interplay of enteric commensal microorganisms and host health and development. Enteric microorganisms are integral in promoting gastrointestinal (GI) tract development, maintaining intestinal epithelial integrity, harvesting and synthesizing nutrients, and promoting development of innate and adaptive immunity through a balance of tolerance to luminal antigens and recognition of pathogens (reviewed in [4]). More recently, the microbial colonization patterns of the airway and more distal lung have been studied to understand how these organisms may be playing a role in respiratory disease. In this review we will discuss some of the emerging data about the interactions of the microbiota of the respiratory and intestinal tracts and how they might be related to lung disease of newborn infants and children.

Definitions

The key terminology used in the field is listed in Table 1 and further discussion can be found in a recent review [5]. Most reports to date have been limited to descriptive studies of the

taxonomic representation of the microbial genomes present based on sequencing of the variable elements of the bacterial 16s ribosomal RNA (rRNA) genes, either V1-3 or V4-6, most commonly. However, deeper metagenomic and metatranscriptomic sequencing have provided significant insights into the function of the microbiota and interactions with the host [6-9].

The respiratory tract microbiome

Previously thought to be sterile, data suggest the presence of bacterial DNA in the lower respiratory tract, some of which is similar to that of the upper airway, but some of which appears to be over-represented in the lower airways [10-12]. Although concerns about acquisition and contamination of lower airway specimens through the necessity of traversing the upper airway in humans are present, careful studies comparing the upper and lower airway microbiota, studies using explanted lungs at the time of transplantation and mouse studies have demonstrated that, although present in low numbers in healthy lungs, there is a lower airway microbial presence that is distinct from that of the upper airway [13-17]. A lingering question remains as to what degree bacterial nucleic acid sequences represent live organisms with the potential to act as pioneering colonizers versus nucleic acid residuals of non-living organisms.

How do the lung microbiota get established?

While patterns of early intestinal colonization following birth have been well characterized [18-20] development of the lung microbiome, particularly the lower respiratory tract, has just recently been examined. The presence of microbiota within the intact uterine-placental environment raises the possibility of colonization even in utero [21, 22]. Postnatal exposures also provide significant influences on microbial colonization patterns. Potential sources include the maternal birth canal, infant skin and intestinal tract and environmental microorganisms, introduced via inhalation, micro-aspiration or in the case of intubated neonates by direct spread. While the basis of initial colonization of the lung is poorly studied, it is known

that the primary determinant of a newborn infant's first microbial community is mode of delivery [23, 24]. Infants delivered vaginally and sampled shortly after birth had microbial communities (skin, oral mucosa, nasopharynx and intestine) most similar in composition to the vaginal communities of mothers, while those born by cesarean section harbored communities more similar to maternal skin and environment. To what extent these pioneering microbiota contribute to lung microbiome is unclear for healthy term neonates.

Animal and human studies would also indicate that the lung microbiome undergoes evolution in the first weeks of life, not unlike what is reported for the intestines. A recent study in mice demonstrated an increasing bacterial load within the first two weeks of life, with a predominance of the phyla Proteobacteria and Firmicutes, followed by subsequent expansion of Bacteroidetes with age [25]. Interestingly a study of 25 preterm infants born at less than 32 weeks of gestation also demonstrated a similar pattern of Proteobacteria (*Acinetobacter spp*) and Firmicutes in tracheal aspirates obtained at birth [26]. These results are consistent with other reports that the amniotic fluid is not a sterile environment, even without rupture of membranes [21, 27]. Other investigators, however, reported low or undetectable bacterial sequences in tracheal aspirates obtained in the first 72 hours from 10 infants less than 28 weeks of gestation [28]. In both these studies of preterm infants though, an evolution of colonization occurred over the first days to weeks of life. In one series, most infants had established a predominant organism by 7 days, either *Staphylococcus sp* (Firmicutes) or *Ureaplasma spp* (Tenericutes) [28] while in the other series, colonization patterns varied by bronchopulmonary dysplasia (BPD) outcome in the second (see "Lung Microbiome And Respiratory Disease" below)[26].

In contrast, studies of healthy term and older infants rely on accessible upper airway samples. Serial analysis of nasopharyngeal samples from 6 weeks to 2 years demonstrated a characteristic early profile where *Staphylococci, Streptococci, Moraxella, Corynebacterium*, or *Corynebacterium* and *Dolosigranulum* predominated at 6 weeks but by 2 years *Moraxella,*

Streptococci and *Haemophilus* predominated. Even the pattern of these samples at 2 years differed from those of adults, where *Moraxella (*Proteobacteria*), Dolosigranulum (*Firmicutes*)* and *Corynebacterium* (Actinobacteria) predominated [29-31].

The adult lung microbiota are primarily represented by the phyla Firmicutes (*Streptococcus* and *Veillonella spp)* and Bacteroidetes (*Prevotella spp*) which comprise about 80% of the species, with lesser representation from Proteobacteria (about10%, *Pseudomonas, Hemophilus* and *Neisseria spp*) [12, 13, 17, 32]. Exactly when the relative proportions of each microbial class achieve the "adult" composition is not clear.

What are the lung microbiota doing?

The more interesting question has to do with the functions that these bacteria, and presumably other microbes, are providing in the lung. There is a complex interaction between the host cells and microbial cells in all microenvironments of the body. In the case of the lung, epidemiologic and experimental evidence suggest a role for local and systemic immune development and regulation (discussed below in "Lung-gut axis"). Epidemiologic observations in the UK suggested a decrease in the prevalence of hayfever with increasing family size, positing the initial "hygiene hypothesis" [33]. Subsequent studies have not borne out the family size contribution but children who have been exposed to farm environments, pets and day care exhibit fewer allergies and asthma, suggesting that early exposure of the airway to allergens or microbial particles may protect against future immunologic or microbial insults [34-36]. Thus, interaction of the airway microbiota and cells of the respiratory tract, including epithelial cells, mucus producing cells or immune effector cells, is likely to influence susceptibility to or protection from disease as well as affect structural development of the lung at critical time points in development [15, 37].

Critical developmental periods

The perinatal period is critical for the programing of immune mediated effects. The importance of microbial exposure during this time period has been elegantly demonstrated in murine models of allergic asthma. Neonatal mice normally demonstrate airway hyper-responsiveness following airway allergen exposure. As the lung bacterial load evolves and increases during development this hyper-responsiveness decreases. If microbial colonization is however, limited during the first two weeks after birth or if the mice are raised to adulthood under germ-free conditions, hyper-responsiveness to airway challenges is maintained with increased airway resistance, elevated serum and tissue IgE levels, and proinflammatory cytokines [38, 39]. These responses have been found to be associated with accumulation of proinflammatory invariant natural killer cells (iNKT) in both the lungs and the intestines and, in other models, they appear to be mediated through a programmed cell death ligand-1 (PD-L1) promoting tolerance to aeroallergens [25, 38]. In the germ-free mice these allergic responses were abrogated only when the mice were recolonized with conventional microbiota through non-organ specific environmental exposures early in life. Recolonization as adults had no effect [38].

The role of the lung microbiota has also been specifically evaluated for its impact on lung development. Bacterial communities were present in the lungs of mice raised under specific pathogen-free (SPF) and non-SPF conditions but not in germ free mice; these communities were more abundant and diverse in non-SPF mice compared to SPF mice. This difference correlated with changes in lung architecture, with the higher bacterial abundance in non-SPF mice correlating with more and smaller alveoli. For confirmation that bacterial colonization was responsible for the observed changes in architecture, germ free mice were inoculated with bacterial isolates early in life, which induced changes in alveolar architecture similar to those observed in non-SPF mice [15].

It is becoming clear that the lung is exposed to bacterial components very early in life and that the perinatal time period is critical in forming these microbial-host interactions that have an impact on lung development and local and systemic immune responses, many of which are modulated through the GI tract.

The "Gut-Lung Axis"

Although interactions between the lung microbiota and respiratory tract cells appear to modulate local immune regulation, development and response, distant interactions with the GI tract may actually be more important in the establishment of local and systemic immune function [40]. Crosstalk between the gut and lung has the potential to exist on multiple levels, from direct physical transfer of bacteria through reflux and micro-aspiration to indirect effects from their byproducts or mucosa-mediated immune responses common to both the GI tract and the lungs [41]. As described earlier, germ-free mice exhibit more severe allergic airway disease and colitis than conventionally raised animals, an effect that can be mitigated by exposure to conventional environmental conditions and flora early in life [38, 42]. Similar effects have been demonstrated in mice treated with enteral antibiotics [43, 44]. Animals treated with clinically relevant doses of vancomycin, but not streptomycin, developed more severe asthma indicating the effect may be more related to microbial composition than numbers. Human epidemiologic studies have also linked shifts in intestinal microbial communities to allergic and asthmatic manifestations [45-47].

The gut microbiota may also affect respiratory function through metabolic by-products, such as short chain fatty acids (SCFA). Mice fed a high fiber diet had increased proportions of Bacteroidetes in the GI tract and higher circulating SCFA. These high fiber-fed animals were protected from allergic airway inflammation, whereas animals fed low fiber diets had increased proportions of Firmicutes, decreased circulating SCFA and increased allergic airway disease

[48]. SCFA also resulted in bone marrow-derived lung dendritic cells that were less capable of driving TH2 cell responses, thus mitigating airway inflammation.

In humans, breast milk feeding affects the composition of both the respiratory and GI tract microbiota, further suggesting a link between the lung and gut [29-31]. Further, serial sampling of respiratory and stool samples in infants with cystic fibrosis demonstrated that, while the microbial communities at the two sites had distinct compositions, there was an overlapping core dominated by *Veillonella* and *Streptococcus,* with a high degree of concordance between bacteria that were increasing and decreasing over time in both compartments. Additionally, dietary changes affected airway microbial composition, suggesting a link between nutrition and the respiratory flora [49].

Thus, the GI tract appears to play a key role in immune development and regulation, some of which may be mediated by nutritional factors, which in turn affects respiratory health and responses to environmental exposures.

Lung microbiome and respiratory disease

Recent studies have started to evaluate the relationship between the airway microbiome and respiratory disease in order to understand its role in the mechanisms or modification thereof and to expand the possibilities for therapeutic intervention. In contrast to studies focusing on the GI tract where the metagenomics have been evaluated, the human lung studies have been primarily limited to descriptive measures of composition, abundance and diversity measures.

Asthma

The strong interactions between the microbiota and immune responses have led to the natural focus on asthma and allergic disease. Studies utilizing bronchoalveolar lavage (BAL) or induced sputum have demonstrated that Proteobacteria (predominantly *Haemophilus spp*) are

more abundant in distal airways of individuals with asthma and chronic obstructive pulmonary disease (COPD); Firmicutes (*Staphylococcus spp*) are more abundant in children with difficult asthma [17, 50, 51]. In contrast, Bacteroidetes, especially *Prevotella spp*, are more abundant in controls [50]. Further suggesting a role for these bacteria in disease are observations from murine and cell-based studies that demonstrated enhanced Toll-like receptor 2 (TLR-2)-independent inflammation for asthma and COPD-associated Proteobacteria (*Haemophilus spp* and *Moraxella*) compared with commensals *Prevotella spp,* which exhibit weak, TLR-2-dependent inflammatory properties [52].

Idiopathic Pulmonary Fibrosis (IPF)

In comparison with healthy individuals who did or did not smoke and individuals with COPD, individuals with IPF had double the bacterial load in their BAL fluid, and higher bacterial load was associated with more progressive disease [53, 54]. Specific species of Firmicutes (*Veillonella* and *Streptococcus*) and Proteobacteria (*Neisseria*) were also associated with IPF after controlling for age and smoking. In addition, the presence of the mucin 5B gene promoter variant minor allele (rs35705950) which is more prevalent in individuals with IPF but is associated with slower progression of disease [55] was also associated with lower bacterial burden raising an interesting speculation about interactions between this genetic variant, burden of bacterial colonization and progression of disease [53]. In contrast, no evidence for microbial dysbiosis was identified in a small group of individuals with non-IPF interstitial pneumonias [56].

Chronic obstructive pulmonary disease (COPD)

As described, many studies have used individuals with COPD as disease controls. The COPD lung resembles that of the asthmatic lung in terms of relative microbial representation, with Proteobacteria and Firmicutes, predominantly *Staphylococci* and *Streptococci*, being abundant [13, 17, 57].

Bronchopulmonary dysplasia (BPD)

Whether or not the microbiome plays a direct role in the pathogenesis of BPD has just begun to be explored. Microbial elements, primarily *Acinetobacter*, could be identified in tracheal aspirates of infants <28 weeks' gestation at birth, even with cesarean delivery. Those who later developed BPD demonstrated decreasing bacterial diversity, decreasing proportions *of Acinetobacter spp* and increasing proportions of *Staphylococcus sp* in tracheal aspirates over the first 3 weeks. Despite these changes in bacterial composition, there was no correlation with inflammatory cytokines, leaving the question of functional importance open [25]. In the study of Mourani, *et al* of a group of infants at risk for BPD, identification of *Ureaplasma spp* as early as 7 days [28] supports past studies suggesting *Ureaplasma i*s a risk factor for development of BPD [58, 59].

Key questions and opportunities

While these human studies have just begun to identify differences in the microbial composition of the upper and lower airways in respiratory health and disease, the functional importance of these differences, if any, remain to be elucidated. Understanding the composition and diversity of this microbiome only touches the surface of the more important issues: do the specific microbiota influence or modify the type of disease, or does the disease environment permit a specific microbial outgrowth? What genes are being expressed by these organisms, what functions are they providing, and how are they interacting with unique cell types, environment, and the host's genome within the different areas of the lung? Applying newly developing high throughput "omics" approaches will provide some of this insight. The challenge for lung disease remains access to the airway, so it will be necessary to identify other more accessible sources that might mimic the respiratory microbiome and can be used as proxies for the distal airway. Finally, the apparent cross-talk between the immune system of the gut and systemic immune

regulation that appears to be determined in early life suggests opportunities for intervention and early prevention.

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Table 1: Common terminology

Microbiome	The collection of microbes and their collective genome, located within a specific habitat.	
Microbiota	The microbial population itself located within a specific habitat.	
16S rRNA gene	Gene that codes for the small ribosomal subunit (16S) of the prokaryotic ribosome, specific to bacteria. It consists of regions conserved between bacteria and hypervariable regions that are unique and used to identify bacterial species.	Amplification and sequence analysis results in identification of nucleic acids that are unique to bacteria.
V1-9	Nine hyper-variable regions of the bacterial 16S rRNA gene, often used in combination to identify the taxa of the bacterial sequence present	Answers the question, “What is the bacterial composition?”
Metagenomics	Sequencing of the entire bacterial chromosome to identify the genes that are present. Provides insight into a microbial community’s functional characteristics	Answers the question, “What is the potential for their activity?”
Meta- - transcriptomics - proteomics - metabolomics	 Identifies genes being expressed Identifies proteins being synthesized Identifies metabolic processes represented	Answers the question, “What are they actually doing?”
Dysbiosis	A state of microbial imbalance, in which the normal microbial community structure has been perturbed, resulting often in disease states.[16]	

Table 2: Classification of bacteria that are commonly associated with health and disease.

Phyla	Actinobacteria	Bacteroidetes	Firmicutes	Proteobacteria	Tenericutes
Genus	*Corynebacteria* *Bifidobacteria*	*Prevotella* *Bacteroides*	*Staphylococci* *Streptococci* *Veillonella* *Dolosigranulum* *Lactobacilli* *Enterococcus*	*Acinetobacter* *Haemophilus* *Neisseria* *Pseudomonas* *Moraxella*	*Ureaplasma*