Current perspectives

The microbial environment and its influence on asthma prevention in early life

Erika von Mutius, MD, MSc *Munich, Germany*

There is accumulating evidence to suggest that the environmental microbiome plays a significant role in asthma development. The very low prevalence of asthma in populations highly exposed to microbial environments (farm children and Amish populations) highlights its preventive potential. This microbial diversity might be necessary to instruct a well-adapted immune response and regulated inflammatory responses to other inhaled and ingested environmental elements, such as allergens, particles, and viruses. Like the internal gut microbiome, which is increasingly recognized as an important instructor of immune maturation, the external environmental microbiome might shape immune responses on the skin, airway mucosal surfaces, and potentially also the gut early in life. The diversity of the external microbial world will ensure that of the many maladapted pathways leading to asthma development, most, if not all, will be counterbalanced. Likewise, important contributors to asthma, such as allergen sensitization and allergic manifestations early in life, are being suppressed. Thus the facets of innate immunity targeted by microbes and their compounds and metabolites might be the master switch to asthma and allergy protection, which has been found in environments rich in microbial exposures. (J Allergy Clin Immunol 2016;■■■:■■■-■■■.)

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Abbreviations used

ALEX: Protection Against the Development of Atopy: Relevant Factors from Farming Environments
aOR: Adjusted odds ratio
EPS: Extracellular polysaccharide
PAMP: Pathogen-associated molecular pattern
PARSIFAL: Prevention of Allergy–Risk Factors for Sensitisation in Children Related to Farming and Anthroposophic Lifestyle

The microbial world is a vast universe with innumerable species. Microbes have been found in the deep sea, caverns, hot springs, and other places not inhabitable by human subjects. However, microbes also populate our daily indoor and outdoor environments in urban and rural areas in great abundance.[1] This abundance had been underestimated as long as culture methods were the only possible means of investigation. Yet isolation and cultivation have strong limitations because for many microbes, it is unknown under which conditions they grow.[2] For certain environments, only about 1% of all microbes can be cultured. Furthermore, cultivation methods are tedious and personnel intensive, which hampers their application to large population-based surveys.

Alternatively, exposure to microbes can indirectly be assessed by detecting substances derived from their cell walls, such as LPS and peptidoglycan. Although LPS or endotoxin is found on the cell walls of gram-negative bacteria, peptidoglycan is a component of the cell wall of gram-positive and also, to a smaller extent, gram-negative bacteria. The most commonly used test (the limulus amebocyte lysate test) to detect endotoxin in dust samples has been largely applied in epidemiologic surveys. Others have measured levels of 3-hydroxy fatty acids from LPS, but levels correlate poorly with limulus amebocyte lysate assay measurements. The same problem applies to different methods detecting muramic acid, a component of peptidoglycan.[3] Other compounds reflecting exposure to fungi, such as extracellular polysaccharides (EPSs) and β- glucans, have also been measured.[4] However, the specificity of these individual markers is limited.

The advent of DNA-based high-throughput analyses has revolutionized the field. These novel study instruments are comparable with the invention of the microscope, which also allowed making the invisible world seen. In 1977, Woese and colleagues[5] discovered that the nucleotide sequence of 16S ribosomal RNA (16S rRNA) is present in every bacterial and archaeal cell but not in human or animal cells. 16S rRNA contains highly conserved regions among all bacteria. Thus detection of 16S rRNA signals the presence of bacteria and archaea but not protozoa, virus, vertebrate, and other animals or human subjects. These conserved regions flank sequence regions that are variable among different bacterial species and thus allow phylogenetic classification. Several techniques combining high-throughput methods, such as microarrays or next-generation sequencing, with 16S rRNA gene analysis are being increasingly applied to microbial investigations of environmental and human samples.[6] The taxonomic classification of the bacteria is obtained

From the Department of Pediatrics, Dr von Hauner Children's Hospital of Ludwig Maximilian University of Munich.

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Corresponding author: Erika von Mutius, MD, MSc, Department of Pediatrics, Dr von Hauner Children's Hospital of Ludwig Maximilian University of Munich, Lindwurmstrasse 4, D-80337 Munich, Germany. E-mail: erika.von.mutius@med.lmu.de.

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by comparing the sequences against databases, such as the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/nucleotide), the Ribosomal Database Project (http://rdp.cme.msu.edu), Greengenes (http://greengenes.lbl.gov/cgi-bin/nph-index.cgi), or SILVA (http://www.arb-silva.de). These techniques are advancing fast, and it is now possible to study all genetic material (human, animal, plant, microbes, and protozoa) recovered from environmental samples by using metagenomic shotgun sequencing. Given the exponential decrease in associated costs, more studies with larger sample sizes will embrace these techniques and provide novel insights into the interplay of these numerous actors.

It seems important to also discuss the limitations of these fantastic techniques. First, it is impossible to know whether the recovered material represents viable microbes, debris, or (most likely) both. The detection of viable bacteria and fungi might not be reflective of important metabolites produced by these microbes. In fact, phylogenetic diversity is probably not translated into functional diversity (ie, diversity of bacterial and fungal metabolites).[7]

These technological advances have allowed the investigation of the microbial world around us but also in and on us in a depth never seen before. The Human Microbiome Project and other studies have shown that the human body harbors 10-fold as many microbial than human cells and orders of magnitude more microbial genes than the human genome.[8,9] The relation between the environmental microbiome, the human microbiome, and the development of childhood asthma and allergies is the theme of this review.

THE ENVIRONMENTAL MICROBIAL WORLD

The fungal and bacterial composition inside and outside of more than 1100 homes across the United States was studied by using novel DNA fingerprinting techniques.[1] Distinct microbial communities were found in indoor and outdoor dust samples, but these differences were larger for bacteria than for fungi.

It has been known that almost all indoor fungi originate from outdoors, except those associated with molds and water damage, wood degrading, and foods.[1] Therefore it is not surprising that an important portion of the variation among homes in fungal community composition was associated with geographic location and regional climatic and land use variables, such as temperature, mean annual precipitation, soil pH, and the diversity of vascular plants.[10] Interestingly, 74% of these fungal taxa were unknown (ie, not represented in the reference sequence databases), highlighting our enormous gap in knowledge about the fungal world.

For bacteria, indoor communities were also found to be distinct from those found outdoors. Only a small proportion (1.6%) of all bacterial phylotypes was significantly more abundant inside homes, but many of these taxa were quite frequent and associated with skin and feces from human occupants.[1] Furthermore, the nonhuman occupants, such as insects, cats, and dogs, determined indoor bacterial composition. More than 50 genera were significantly more abundant in homes with dogs or cats, including members of the *Prevotella*, *Porphyromonas*, *Moraxella*, and *Bacteroides* genera, which occur in the mouths or feces of dogs and cats.[1] These bacterial phylotypes were so specific that they allowed prediction of dog and cat ownership with 92% and 83% accuracy, respectively. The influence of pets on indoor bacterial community structure has been shown repeatedly,[11,12] but day care attendance might also play a role.[11] Thus the presence, identity, and activities of home occupants are significant determinants of indoor bacterial composition in addition to a relevant input from outdoor sources, which relate to soil and climatic variables. Interestingly, the microbiome of outdoor sources was not affected by urbanization.[7]

Studies using endotoxin as a proxy for bacterial exposures have come to similar conclusions. For example, the Effects of Outdoor and Indoor Air Pollution on the Development of Allergic Disease in Children (AIRALLERG) study in 3 European countries concluded that keeping pets and having more than 4 persons living in the home were consistently associated with higher indoor endotoxin concentrations.[13] Also, day care centers have high levels of endotoxin,[14] and indoor storage of organic household waste increases endotoxin and fungal markers.[15] The input from outdoor environments rich in bacterial exposures, such as farms, has been documented by using conventional culture methods and endotoxin measurements.[16] Bacteria and fungi were more often detected and grown in higher quantities from farm children's bedrooms than bedrooms of children not exposed to farm activities. The microbial counts in farm children's bedrooms correlated with the counts in animal sheds. Similar findings have been reported for endotoxin,[17] suggesting a significant transfer of bacteria from outdoor to indoor environments.

THE LUNG MICROBIOME

The new DNA-sequencing methods have shown not only that the indoor and outdoor air that we breathe daily contains thousands of microorganisms but also that these microbes actually enter and potentially colonize the lower airways. Hilty et al[18] were among the first to demonstrate that in healthy children and adults bacteria are found in bronchoalveolar lavage fluid and airway brushings, respectively. The bacterial composition differed between healthy subjects and asthmatic patients in whom more Proteobacteria, in particular *Haemophilus influenzae*, were found. Lower airway samples can be contaminated by passage of a bronchoscope through the oropharynx. More recent studies suggest that the lower airway microbiome resembles a dilution of the upper airways and oral cavity microbiome. However, specific bacteria were detected in significantly higher abundance in the lungs (Enterobacteriaceae and *Haemophilus* species among other), suggesting also a unique airway microbiome.[19,20] Other investigators have supported the notion of a greater representation of Proteobacteria, including *H influenzae* and *Pseudomonas*, *Neisseria*, and *Burkholderia* species, as well as Enterobacteriaceae, among asthmatic patients.[21] These associations were independent of age,[22] steroid treatment, and asthma severity.[23,24]

Although these data all suggest a role for Proteobacteria in asthmatic patients, the hen-egg problem has not been solved. Inflammation in the airways of asthmatic patients might create a niche promoting the growth of these bacteria very early in life. In turn, colonization with certain bacteria can promote the development of asthma. In the COPSAC birth cohort the detection of *H influenzae*, *Moraxella catarrhalis*, and *Streptococcus pneumoniae* by means of conventional culture methods in the hypopharynx of 1-month-old infants preceded the development of persistent wheeze and asthma, as defined prospectively by the age of 6 years.[25] However, in the same cohort this asymptomatic colonization was also associated with increased risk of