Published in final edited form as:

Early Hum Dev. 2010 July ; 86(Suppl 1): 67–71. doi:10.1016/j.earlhumdev.2010.01.018.

The Infant Intestinal Microbiome: Friend or Foe?

Maka Mshvildadze^{1,2} and Josef Neu²

¹Chachava Scientific-Research Institute of Perinatal Medicine Obstetrics and Gynecology, Tbilisi, Georgia

²Department of Pediatrics, University of Florida, Gainesville, Florida, USA

Abstract

During the course of mammalian evolution there has been a close relationship between microbes residing in the gastrointestinal (GI) tract and the mammalian host. Interactions of resident intestinal microbes with the luminal contents and the mucosal surface play important roles in normal intestinal development, nutrition and adaptive innate immunity. The GI tract of the premature infant has a large but fragile surface area covered by a thin monolayer of epithelial cells that overlies a highly immunoreactive submucosa. Interactions in the lumen between microbes, nutrients and the intestinal mucosa can range from a healthy homeostasis to an uncontrolled systemic inflammatory response syndrome (SIRS) that leads to multiple organ system failure and death. Recent advances in molecular microbiota analytic methodology that stem from advances in high throughput sequencing technology have provided us with the tools to determine the GI microbiota in great depth, including the nearly 80 % of microbes in the intestine that are very difficult if not impossible to culture by current methodology. Application of these techniques to derive a better understanding of the developing intestinal ecosystem in the premature neonate may hold the key to understand and eventually prevent several important diseases including necrotizing enterocolitis (NEC) and late onset neonatal sepsis that may be acquired via translocation through the GI tract.

Keywords

microbiota; microbiome; intestine; premature infant; necrotizing enterocolitis

Introduction

Humans have been associated with complex microbial communities throughout evolution. The human genome project has been highly instrumental in developing new technologies to explore these communities. They are demonstrating that the largest and most complex of the human microbial populations is located in the intestinal tract.¹ During adulthood the intestinal microbiota (IM) is composed of over 400 species and the total number reaches approximately 10^{14} . The IM comprises 10 times more microbial cells than human cells, and 100 times genes present in the human genome. The composition of the intestinal microbiota

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Correspondence to J. Neu, Department of Pediatrics, University of Florida, 1600 S.W. Archer Road, Human Development Building Room HD 513, Gainesville, FL 32610, USA. neu@peds.ufl.edu, Phone: +1-352-392-3020, Fax: +1-352-846-3937.

Statement of Disclosure: We declare that we have no conflicts of interest.

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has been found to be diverse and very dynamic. Adequate knowledge of the types of microorganisms as well as the events that influence the timing of colonization may provide opportunities to modulate the microbiota when needed to prevent or treat disease and improve overall health.

To understand the range of human genetic and physiologic diversity, the microbiome and the factors that influence the distribution and evolution of the constituent microorganism must be characterized. This is the main goal of the Human Microbiome Project (HMP), which is a logical extension of the Human genome Project, largely based on newly developed technologies from the latter.²

One can consider the IM as an essential organ in providing nourishment, regulating epithelial development and instructing innate immunity. Characterization of this immensely diverse ecosystem is the first step in elucidating its role in health and disease. Advances in molecular microbiota analytic methodology that stem from advances in high throughput sequencing technology are now providing us with the tools to determine the GI microbiota in great depth. Molecular approaches that identify microorganisms from small-subunit (16S) ribosomal RNA genes sequences offer advantages over cultivation. The 16S rRNA gene is typically chosen because it is present universally and can provide a taxonomic identification ranging from domain and phylum level to approximately the species level. The rRNA gene sequences comprise highly conserved sequence domains interspersed with more variable regions and simple profiling approaches such as Denaturing Gradient Gel Electrophoresis (DGGE), Restriction Fragment Length polymorphisms (RFLP) and Automated ribosomal Intergenic Spacer Analysis (ARISA) allow for an efficient evaluation of overall microbiota diversity. These profiling methods are often complemented with quantitative tools such as fluorescent *in situ* hybridization (FISH) and real time PCR. Recently, powerful novel sequencing approaches, including pyrosequencing, allow for an in depth analysis of even minor members of the microbiota.³ However, these methods have been used to study human microbiota ecology for only a decade and the available data are limited.

Intestinal Colonization in the Infant

Early microbial colonization of the intestine

Existing literature suggests that the neonatal intestine is sterile at birth when culture based techniques are used to analyze meconium. During birth and shortly thereafter, microbes form mother and the surrounding environment colonize the GI tract of the infant and a dense, complex microbiota develops. Currently little data exists about microbial DNA in the first stools of term or premature babies using non-culture based methods. Previous studies have demonstrated the presence of microbes in amniotic fluid without rupture of membranes using both culture and non culture based techniques.^{4,5} In one of these, a correlation was found between extent of microbial colonization and length of gestation.⁴ A strong body of evidence suggest that occult intra-uterine infection plays a major role in preterm labor and delivery. These infections are thought to escape detection primarily because they are subclinical, but also because they may be caused by cultivation –resistant microbes. However molecular studies that define the diversity and abundance of microbes invading the amniotic cavity and evaluate their clinical significance are lacking. In recent study DiGiulio et al.⁴ found the amniotic cavity of women in preterm labor harbors DNA from a greater diversity of microbes than previously suspected, including as yet uncultivated, previously uncharacterized taxa. A recent study by our group was the first to find microbial DNA in the first stools (meconium) of premature babies less than 32wk GA using non-culture based methods.⁶ We speculate that microbial DNA in meconium of premature infants suggests prenatal origin. About 50% of amniotic fluid volume is swallowed by the fetus daily,⁷ thus amniotic fluid containing microbes may have been swallowed in utero and colonization of

the GI tract might start prenatally. According to the findings of DiGiulio,⁴ increasing degree of prematurity is associated with more amniotic fluid microbial colonization, then one would also expect to find more microbes in the first stools of the more premature infants.

Microbial -Intestinal interactions

The gastrointestinal tract (GI) of the premature infant has a large but fragile surface area covered by a thin monolayer of epithelial cells that overlies a highly immunoreactive submucosa. Interactions in the lumen between microbes, nutrients and the intestinal mucosa can range from a healthy homeostasis to an uncontrolled systemic inflammatory response syndrome (SIRS) that leads to multiple organ system failure.

The preterm infant is particularly sensitive to colonization patterns as inherent intestinal defense mechanisms are immature and immature intestinal epithelial cells are known to have exaggerated inflammatory responses to both commensal and pathogenic bacteria.⁸ With the core microbiota formation being dependent on exposure to the microbes that first colonize the GI tract the establishment of a “healthy” microbiota in the first several days of life after birth is likely to be critical for normal development. Thus factors that effect the microbiome exposure such as mode of delivery, use of antibiotics, delayed enteral feeding or living in a neonatal intensive care unit with a potentially high load of pathogens, exposure of the babies mothers oral and skin microbiota and breast milk versus of the formula ingestion, are likely to play a important roles. The core microbiota once developed in the individual, may be difficult to eradicate or modify. Whether the infant is born prematurely and requires intensive care versus term infant who is born without special needs could markedly affect the development of the intestinal microbial core.⁹

Changes in the IM composition/activities and host cell gene expression affect the developing intestine and can affect the efficiency of nutrient uptake. Reduction of the normal commensal microbiota diversity due to overgrowth by infectious agents, delayed enteral feeding or antibiotic treatment may thus interfere with the availability of critical nutrients and impair beneficial stimulation of GI mucosal development and the innate and adaptive immune responses.¹⁰

Establishment of the IM under different conditions

During the birth process and rapidly thereafter microbes from mother and the surrounding environment colonize the GI tract of the infant eventually leading to a dense and diverse bacterial community.¹¹ This colonization is a complex process that appears to be influenced by the host microbe interactions as well as internal and external exposures such as type of feeding (breast versus formula) and mode of the delivery (caesarian versus vaginal)^{12,13} antibiotics given to the mother or infants, prolonged rupture of membranes, gestational age or the birth weight at birth.

Previous studies using primarily culture-based techniques have suggested differences in microbial colonization of breast versus formula fed infants, but this remains controversial.¹⁴ Several studies found a lower abundance of Bifidobacteria and a higher abundance of aerobic bacteria in the GI microbiota of formula-fed infants relative to breast-fed infants,^{24,25} yet other reports have found no such difference. One study compared the IM of breast fed with formula fed preterm infants using 16S gel techniques,¹⁵ and showed that breast fed infants have more diverse bacterial population than formula fed infants with lower numbers of pathogenic microorganisms.¹⁶ Studies have also shown that microbes and their cellular products are present in human mother's milk, which may represent translocation through her GI tract and blood-stream into the breast milk.¹⁷ The implications of this remain unclear, but it is speculated that they may offer a protective effect for the neonate by

interaction with intestinal cellular receptors that modulate the inflammatory response.⁹ Additional comprehensive non culture based analyses are needed to provide the critical data required to evaluate differences in IM in formula versus breast fed premature infants.

The use of broad spectrum antibiotics in pediatric practices alters the gut colonization and consequently may impair the barrier function.¹⁸ Little is known about how antibiotics alter the establishment of GI microbiota in premature infants. Several studies report colonization with lactobacilli is delayed with antibiotic treatment. However, most of the studies used cultivation based techniques.^{16,17,22}

Studies evaluating the response of intestinal development in rodents after antibiotic administration showed that neonatal antibiotic treatment alters gastrointestinal tract development gene expression and the intestinal barrier transcriptome. Daily intragastric gavage treatment with Clamoxyl resulted in the near complete eradication of *Lactobacillus* in the intestine and in a drastic reduction of colonic total aerobic and anaerobic bacteria, in particular *Enterobacteria* and *Enterococci*, but this was also associated with a marked alteration in numerous gene expression patterns including the MCH genes, which are involved in subsequent immune tolerance.²¹ A recent study by our group using molecular approaches⁶ report also that antibiotics given to the mother antepartum reduces IM diversity in the first stool samples, and after the first several samples the diversity differences between control and maternal antibiotic treated infants disappeared.

The fact that antibiotics are routinely used in premature infants should raise alarm and begs for future studies in this area. In retrospective cohort analyses of extremely low birth weight infants Cotton et al. found that prolonged initial empiric antibiotic therapy was associated with increased risk of NEC and death and should be used with caution.²²

Role of the IM and the Innate Immune System

The Toll Receptor System

Components of bacteria provide chemical signals that are recognized by specific receptors – called toll-like receptors (TLRs) - of the innate immune system. The mechanisms of how microbes or their components interact with this system to provide innate immunity are still in the early phases of research. It is assumed that the healthy intestinal surface somehow defuses the threat of commensal bacteria to the lumen where they thus reside undetected. A study by Rakoff-nahoum et al.²³ provides insights how this happens: commensal bacteria interact with the intestinal surface and to some degree trigger TLR signaling. The authors used mice deficient in either TLR-2 or TLR-4 or a necessary downstream component of the TLR pathway (MyD88), to prevent TLR-signaling. Such mice were shown to have a profoundly exaggerated response to intestinal injury. This effect is not a consequence of acute inflammation or the unrestrained overgrowth of commensal bacteria; rather, it results from the loss of TLR-dependent conditioning that allows the intestinal surface to maintain its normally resistant homeostasis. Surprisingly this interaction is required to maintain the architectural integrity of the intestinal surface. Thus it seems that the epithelial and resident immune cells do not simply tolerate commensal bacteria but are dependent on them.²⁴

Toll-like receptors (TLRs) are transmembrane proteins that are found on the surface of a variety of host defense cells. TLRs recognize specific pathogen regions or molecular motifs, referred to as pathogen-associated molecular patterns (PAMPs), which have been conserved over time. Therefore, TLRs represent a component of the host's innate immune defense. To date, over 10 different TLRs have been identified, each having a specific family of PAMPs for which it binds. It is now known that human TLR-2 and TLR-4 are expressed on the surface of crypt enterocytes and to a lesser degree, on the surface of microvillous and villous

epithelium. The identification of TLRs on enterocytes helps explain the mechanism by which commensal and pathogenic bacteria participate in microbial-epithelial cross talk to regulate the intestinal inflammatory response. The binding of a PAMP-specific region to its corresponding TLR results in modulation of the inflammatory cascade by way of nuclear factor- κ B (NF- κ B). When activated, NF- κ B translocates into the cell's nucleus and switches on the genes responsible for the expression of inflammatory mediators. It has been shown that in the mature enterocyte, commensal bacteria may exert its beneficial effect by inhibiting the activation of NF- κ B; this is in contrast to pathogenic bacteria and associated antigens (lipopolysaccharide-LPS) which activate NF- κ B. Of particular importance to the premature neonate, is that the expression of inhibitory factor kappa B (I κ B), a protein which, when bound to NF- κ B inhibits NF- κ B translocation into the cell nucleus, is developmentally regulated. I κ B expression is reduced in immature fetal intestinal cells. As a result, immature epithelial cells demonstrate excessive inflammation when the TLR pathway is activated and this excessive inflammatory response is seen with both commensal and pathogenic bacteria.²⁵ Moreover, the regulation of other TLR negative signal intermediates, which inhibit inflammatory activation, is downregulated during fetal development and expression of these intermediates is further reduced with NEC as demonstrated by microarray analysis of intestinal samples.²⁶

Quorum sensing and Biofilms

Individual bacteria communicate with each other and coordinate group activities using cell signaling mechanisms where the populations responds when a critical number is reached, in a process termed quorum sensing. Conversion of a microbial community from planktonic to biofilm form may result in markedly different properties and physiologic properties for the same type of bacteria. When a certain number of bacteria are reached, the microbes themselves may display a different physiologic profile than before the quorum was formed.²⁷ The relationship of microbial quorums and their behavior in health and disease are fertile areas for investigation.

Angiogenesis

Studies on animal model showed that indigenous microbes act as environmental agents that shape the development of the intestinal villus microvasculature through the Paneth cells. These cells are strategically positioned at the base of the crypt to coordinate development of the both the microbiota and the microvasculature.²⁸ Comparison of the capillary network of germ-free mice with ex-germ-free animals colonized after completion of postnatal gut development showed arrested capillary network development in the germ-free animals that restarted and was completed within 10 days after colonization with a complete microbiota harvested from a fully developed animal. These findings showed that microbial colonization regulates the microvasculature by signaling through a bacteria-sensing epithelial cell.²⁸

Control of Inflammation in the Gut: the “old friends” hypothesis

The intestinal epithelium must discriminate between pathogens and nonpathogenic organism as well as food antigens. It must be tolerate the commensal flora present in the lumen and maintain mucosal homeostasis by controlling inflammatory responses, as well as sensing the danger signals of potentially harmful pathogens. The “old friend” hypothesis states that the presence of normal microbes (“old friends”) stimulates a low grade up regulation of T regulatory cells that produce IL-10 and transforming growth factor- β (TGF β), which in turns modulates the effect of proinflammatory process.²⁹ In addition to tolerating the old friends, this mechanism also maintains tolerance to “self”. This is likely to be one of the critical mechanisms underlying the benefits of maintaining GI commensal microorganisms as well as supplementation with probiotics.

Role of IM in disease

Intestinal Microbial colonization during necrotizing enterocolitis (NEC), systemic inflammation (SIRS) and sepsis

The thesis that intestinal microbes are necessary for the development of NEC is supported by several lines of evidence.³⁰ These include epidemiologic evidence for outbreaks suggestive of an infective process, the frequent isolation of infectious agents such as *Klebsiella* and *E.coli* with NEC, and decreased incidence of NEC resulting from preventive antibiotic measures. Also the gas observed in pneumatosis intestinalis one of the harbingers of NEC is thought to be derived from bacterial fermentation. The large diversity of bacteria associated with NEC suggests that they might be bystanders that amplify unrelated other processes, such as inflammation.

The majority of the very premature infants in the NICU are started on broad spectrum antibiotic therapy shortly after birth during the rule out sepsis work-up;³¹ this can alter normal flora with which the neonate would become colonized. Rather than becoming colonized with potentially beneficial microbes that include various strains of lactic acid bacteria and *Bifidobacteria*, antibiotic resistant species indigenous to the NICU may colonize the premature infants' intestine.³² Such pathogenic microorganisms have a greater propensity to activate cell surface receptors that transduce signaling molecules such as nuclear factor kappa B which incite a pro-inflammatory response via the synthesis of pro-inflammatory cyto- and chemokines.³³

At this juncture, no specific bacteria or bacterial pattern has been causally associated with the development of NEC. Despite this, the development of microbial patterns with altered diversity or the formation of blooms that reach a quorum to pathogenicity may become of importance. Recent studies using molecular techniques have provided complementary information.³⁴ Using a 16S rRNA based PCR approach Millar *et al.* studied bowel flora of preterm infants to determine the extent to which bacteria not detected by culture contribute to the microbial flora of the bowel of preterm infants with and without NEC.³⁵ In this study uncultured bacteria detected by PCR-DGGE were no more frequent in the fecal samples from infants with NEC than in infants without NEC. These findings in feces do not exclude the possibility that as of yet unrecognized bacteria might be associated with the mucosa associated microbiota of the small intestine of infants with NEC. In the Millar study stool collection did not start until after clinical symptoms of NEC occurred. In addition antibiotic therapy frequently had started before sampling. This might have distorted differences in microbiota before the onset of disease and/or treatment and may limit the applicability of the results of this study.

A potential association between early colonization by *Clostridium* and NEC was described in a prospective case control study in infants at 34 weeks of gestational age.³⁶ Using TGGE profiling the authors detected colonization by *Clostridium* that occurred before the development of NEC in all cases. The molecular band representing *Clostridium* was not detected in any of the nine controls.

The Gut is the Motor that Drives Systemic Inflammation and Multiple Organ Dysfunction

The large diversity of bacteria associated with NEC suggests that they might be bystanders that amplify unrelated other processes, such as inflammation. In an intensive care nursery, premature infants acquire commensals slowly, and the establishment of commensals such as *Bifidobacterial* flora is retarded. A delayed bacterial commensal colonization of the gut tends to render bacterial species virulent, which upregulates signals to TLR-4. This abnormal upregulation of TLR-4 is associated with activation of NFκB promoting the

transcription of genes for inflammation, and also with increased levels of inducible nitric oxide synthase (iNOS), another potent pro-inflammatory regulator.

Similar to sepsis and adult respiratory stress syndrome NEC seems to involve a final common pathway that includes the endogenous production of inflammatory mediators in the development of the intestinal injury: Endotoxin lipopolysaccharide (LPS), platelet activating factor (PAF), tumor necrosis factor (TNF) and other cytokines together with prostaglandin and leukotrienes and nitric oxide.³⁰

Although the link between disorganized IM and NEC is still evolving, ample evidence from animal studies has accumulated pointing towards activation of inflammatory pathways possibly by abnormal IM in NEC infants vs activation of normally suppressed immune pathways (commensal mediated) promoting tolerance and immunity.^{37,38}

Recent findings highlight the promise of new molecular microbiota typing techniques for the detection of contributions of gut microbiota to NEC in future large scale studies. A better understanding of associations between early intestinal microbial colonization, production of short chain fatty acids (SCFA) which may be either protective or injurious in premature infants, innate immunity and NEC should facilitate development of screening tests for the early detection of the infants at high NEC risk to help in the choice of preventive treatment.

Caveats to the manipulation of the intestinal microbiome in early infancy

Antibiotics are increasingly prescribed in the both the antenatal and neonatal periods. In the NICU widespread use of antibiotics parenteral nutrition and feeding in the incubators and radiant warmers, long parenteral nutrition, delayed feeding may also delay or impair the normal colonization of the intestine. The organisms initially colonizing the gut may establish chronic persistence in many children, in contrast to effective and prompt clearance if encountered in later infancy, childhood or adulthood. Individual members of the gut flora specifically induce gene activation within the host modulate mucosal and systemic immune function and likely have an additional impact on metabolic programming during this highly vulnerable period.³⁸ This clearly raises the specter of lifelong consequences when the microbiome is manipulated in early life and any therapeutic or preventative approaches that alter the intestinal microbiome in early infancy should proceed with a high level of caution.

Conclusion

New information about the importance of normal establishment and maintenance of the intestinal ecosystem during the immediate neonatal period and early life is emerging. Perturbation in this ecosystem especially during early infancy may have consequences that extend well beyond the neonatal period and manifest as diseases in the later life. Although it is tempting to rebuild the intestinal microbiota with use the agents such as pro and prebiotics during infancy, a caution approach on the basis of sound scientific data is warranted.

Acknowledgments

This work was partly supported by the National Institute of Research Resources, National Institutes of Health; grant numbers: RO1 HD 059143 and M01RR00082 and an educational grant to M. Mshvildadze from the European Society for Pediatric Research.

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