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The role of the intestinal microbiota in the pathogenesis of necrotizing enterocolitis

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ABSTRACT

Development of necrotizing enterocolitis (NEC) requires a susceptible host, typically a premature infant or an infant with congenital heart disease, enteral feedings and bacterial colonization. Although there is little doubt that microbes are critically involved in the pathogenesis of NEC, the identity of specific causative pathogens remains elusive. Unlike established normal adult gut microbiota, which is quite complex, uniform, and stable, early postnatal bacterial populations are simple, diverse, and fluid. These properties complicate studies aimed at elucidating characteristics of the gut microbiome that may play a role in the pathogenesis of NEC. A broad variety of bacterial, viral, and fungal species have been implicated in both clinical and experimental NEC. Frequently, however, the same species have also been found in physiologically matched healthy individuals. Clustered outbreaks of NEC, in which the same strain of a suspected pathogen is detected in several patients suggest, but do not prove, a causative relationship between the specific pathogen and the disease. Studies in *Cronobacter sakazakii*, the best characterized NEC pathogen, have demonstrated that virulence is not a property of a bacterial species as a whole, but rather a characteristic of certain strains, which may explain why the same species can be pathogenic or non-pathogenic. The fact that a given microbe may be innocuous in a full-term, yet pathogenic in a pre-term infant has led to the idea of opportunistic pathogens in NEC. Progress in understanding the infectious nature of NEC may require identifying specific pathogenic strains and unambiguously establishing their virulence in animal models.

Introduction

Although the exact etiology of NEC remains elusive, multiple risk factors have been implicated in the pathogenesis of the disease.[1] NEC develops in a susceptible host, typically a premature infant or an infant with congenital heart disease. Such patients often suffer from hypoxia, hypothermia, or general physiologic impairment. Their mucosal immune system is immature or underdeveloped and unable to adequately respond to incoming microbes. Enteral feeding, especially the administration of infant formula, constitutes another important risk factor. Formula, which lacks the protective factors normally found in breast milk, further predisposes the immature intestinal epithelium to injury. With enteral feeding comes microbial colonization of the gut. Some of the colonizing species are beneficial normal commensals, but others are capable of inflicting damage to the intestinal epithelium, especially in the setting of gut ischemia. When such harmful microbes abound, they can cause mucosal inflammation, which results in the production of high levels of inflammatory factors, including cytokines, nitric oxide, platelet-activating factor, and prostanoids to name a few, which further damage the epithelial barrier. Bacterial translocation across the compromised barrier exacerbates the inflammatory response, leading to more epithelial damage, more bacterial translocation, and ultimately, intestinal necrosis. Current research in NEC is aimed at elucidating the mechanisms underpinning specific aspects of this broadly accepted pathophysiologic scenario.[2] This review focuses on the role of the intestinal microbiota in the pathogenesis of NEC.

Early microbiota and NEC

Early microbiota

Culture-based methods, metabolite analysis, 16S rDNA analysis and their combinations have been employed to characterize bacterial populations in neonates of different gestational and/or postnatal ages. These same techniques have also been employed to define bacterial populations in neonates with different disease status subjected to various feeding regimens. When such diverse

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methods have been used in the same study, they have yielded largely similar results.[3–9] Interestingly, little or no unculturable species of bacteria have been found, which supports the idea that culture methods miss relatively few, if any, bacterial species when it comes to analysis of the early microbiota.[10] Most of the studies examined fecal microbes. On occasion, however, mucosal biopsies and luminal content were also analyzed, and the results suggested or confirmed the adequacy of fecal sampling.[5,11,12] These studies established three important characteristics of the early microbiota: low complexity, high inter-individual diversity, and fluidity.

Low complexity

Established adult microbiota consists of over a thousand bacterial species.[13,14] By contrast, the number of major bacterial species colonizing the gut during the early postnatal period rarely exceeds 10. In five temporally and geographically independent studies covering a total of 134 neonates, the average number of major bacterial species per fecal sample was about 3 and the range was 1–8.[6,10,15–17] High-resolution pyrosequencing analysis of 16S rDNA revealed higher levels of diversity; however, most of the species detected represented less than 1% of the total population. The composition of these minor species did not follow any particular pattern and was unique in each neonate.[18] Wherever relative abundance of bacterial species has been reported, usually there has been one predominant species in each sample.[15,17] In fact, low complexity has been consistently reported in numerous studies of early microbiota in humans and animals over the past 40 years.

High inter-individual diversity

Whereas patterns of adult microbiota are structured and conserved,[19] emerging microbial populations of the neonates are very dissimilar. Extreme diversity of early populations has been found in essentially all studies. Overall, more than 85 different species of microbes have been reported as dominant colonizers, alone or in various combinations (Table 1). The most frequently found colonizers are staphylococci, *Escherichia coli*, enterococci, clostridia, *Bacteroides*, and streptococci. These species are omnipresent and abundant normal commensals of skin, GI tract, or oral cavity; therefore it is not surprising that neonates frequently acquire them through parental contact. Less frequent is colonization with other common human commensals such as *Enterobacter*, *Klebsiella*, *Shigella*, *Haemophilus*, *Pseudomonas*, *Lactobacillus*, *Veillonella*, *Bifidobacterium*, and *Atopobium*. A surprising finding is that early human intestinal microbiomes may sometimes be dominated by species that are not typically human commensals, for example bacteria from soil (*Klebsiella oxytoca*, *Acinetobacter*), plants (*Pantoea*, *Raoultella*, *Klebsiella planticola*), water (*Rahnella aquatilis*), domestic animals (*Enterococcus raffinosus*, *Enterococcus avium*, *Enterococcus gallinarum*, *Streptococcus bovis*) and other sources (*Enterobacter asburiae*, *Cronobacter sakazakii*, *Serratia*, *Morganella*, *Ewingella*, *Citrobacter*, *R. productus*, *Weissella*, and *Corynebacterium*). It is possible that the list in Table 1 is only the tip of the iceberg, given the plethora of bacterial species that can become dominant first colonizers. Our studies in newborn formula-fed neonatal rats indicate that littermates handled and fed the same way may nevertheless have very dissimilar patterns of colonization during the first few days of life (Bell et al., unpublished data). Extreme diversity of first colonizers suggests that the naive neonatal GI tract constitutes an attractive or welcoming venue for a broad variety of microbes. Another important conclusion is that initial microbial colonization is accidental; the species that enters the intestine first and in greatest numbers establishes itself as the first colonizer. Although normal human commensals are more likely to be first colonizers, sometimes bacteria from the environment may get there first. Initial colonization diversity is manifested not only in the variety of species, but in the variety of numbers as well. Population densities of 10^3–10^{10} cfu/g of feces have been reported.[3,11,20–22]

Fluidity

In a number of studies, temporal changes in the intestinal microbiota have been traced in the same infant. These studies consistently found that regardless of what bacterial species appear first, there are relatively rapid changes in species diversity and overall load.[3,5–7,9,11,16,18,21,23–25] The first dominant colonizers are usually "outcompeted" by other species during the first 7–10 days of life. These new species in turn are overtaken by different groups of bacteria within a matter of weeks, and this succession pattern continues until the adult microbiota is established at about 1 year of age. In fact, the diversity of species and overall bacterial loads continue to increase over time. The remarkable similarity of succession patterns in a case of twins suggests that succession steps in each individual may be strongly influenced by incidental environmental exposure.[21] As all proverbial roads lead to Rome, all initial colonization patterns lead to the adult pattern via individualized succession steps.

Intestinal microbiota and NEC

Since susceptibility to NEC temporally coincides with the period of extremely diverse microbiota, it is reasonable to suggest that this disease is associated with certain colonization patterns. To elucidate NEC-specific colonization patterns, a variety of studies compared intestinal microbiota in pre-term vs. full-term, breast-fed vs. formula-fed, and NEC vs. non-NEC infants, as well as microbiota in neonates delivered vaginally vs. by cesarean section. There were little differences between vaginally delivered pre-term and full-term infants except for delayed colonization, relative paucity of commensal anerobes, and lower species diversity in the pre-term infants. These differences could be attributed to lower food intake by pre-term infants.[3,6,9–11,16,18,22,25] Neonates delivered via cesarean section were less likely to be colonized by *E. coli*, *Bacteroides*, and *Bifidobacterium* than those delivered vaginally. Conversely, they were more likely to be colonized with clostridia and environmental bacteria than those delivered vaginally.[4,23] Breast-fed babies displayed remarkably faster colonization with *Bifidobacterium* and *Bacteroides*,[22,25,26] although a different study did not support this finding.[9] In some studies, NEC was associated with overabundance of non-*E. coli* Gram-negatives,[17,20,27–29] clostridia,[29–31] *Enterococcus*,[4,32] *Staphylococcus*,[32] *Candida albicans*,[4] *Lactobacillus*,[20] and lower species diversity.[17] Other studies failed to establish any correlation between NEC and intestinal microbiota, prompting some authors to conclude that NEC is not associated with any infectious agent.[5,7,33–37] Since the same bacterial species were found in both NEC patients and in healthy controls, no conclusion could be drawn as to the identity of the causative pathogens in infants who develop NEC. Although microbiome-wide studies might have revealed some patterns associated with NEC, they failed to unambiguously establish a relationship between the disease and the microbiota.

Clinical data

A broad variety of bacterial, viral, and fungal species have been implicated in both clinical and experimental NEC. Clustered outbreaks of NEC, in which the same strain of a suspected pathogen is detected in several patients suggest, but do not prove, a causative relationship between the specific pathogen and the

Table 1
First colonizers isolated from stools of 1–10-day-old infants

Systematic groups	Species	Ecology	Potential opportunistic pathogen	References
Proteobacteria				
Enterobacteriaceae	*E. coli*	Gut commensal	Yes	4,5,7,9–11,16,17,20,23,24
	Enterobacter sp.			4–6,17,22,23,28
	E. cloacae	Gut commensal, environment	Yes	5,7,9,10,16
	E. aerogenes	Gut commensal, environment	?	9,16
	E. asburiae	Environment	Yes	24
	Pantoea agglomerans	Plants, insects	Yes	5
	Serratia sp.			4,16
	S. liquefaciens	Environment	Yes	5
	Shigella sp.	Primate gut commensal	Yes	17
	Klebsiella sp.			4,11,17,20
	K. pneumoniae	Gut, oral, skin commensal; environment	Yes	3,5,9,10,28
	K. oxytoca	Soil	Yes	5,10,31
	Raoultella planticola	Plants	Yes	10,23
	Proteus sp.			7,11
	P. mirabilis	Gut commensal, environment	Yes	4
	Morganella morganii	Environment	Yes	4,5
	Rahnella aquatilis	Environment	Yes	5
	Ewingella americana	Environment	Yes	7
	Citrobacter sp.			16
	C. freundii	Environment	No	4,5,9
	Cronobacter muytjensii	Environment	?	28
Pasteurellaceae	*Haemophilus* sp.	Airway commensal	Yes	17
	H. parainfluenzae	Airway commensal	Yes	5
Moraxellaceae	*Acinetobacter* sp.	Soil	Yes	4
Pseudomonadaceae	*Pseudomonas* sp.			4,5,11,17,20
	P. aeruginosa	Environment, gut commensal	Yes	7,9,16
Firmicutes				
Enterococcaceae	*Enterococcus* sp.			3–7,9,15,17,20,22,23
	E. faecalis	Major gut commensal	Yes	10,16
	E. faecium	Major gut commensal	?	10,16
	E. gallinarum	Gut commensal of birds	Yes	10
	E. avium	Gut commensal of birds	Yes	24
	E. raffinosus	Gut commensal of animals	Yes	24
Closrtidiaceae	*Clostridium* sp.			3,4,6,7,15,22,25,31
	C. perfringens	Ubiquitous	Yes	11,31
	C. butyricum	Soil, gut commensal	Yes	10,11
	C. difficile	Soil	Yes	10,11
	C. neonatale	Environment	Yes	10
	C. disporicum	Environment	?	24
	C. paraputrificum	Environment	Yes	24
	Ruminococcus gnavus	Gut commensal	?	24
	R. productus	Environment	?	10
Staphylococcaceae	*Staphylococcus* sp.			3,4,6,7,17,20,22,25
	S. aureus	Skin commensal, soil	Yes	9,11,16,23,31
	S. haemolyticus	Skin commensal	?	16
	S. epidermidis	Skin commensal	Yes	9–11,16,28,31
Streptococcaceae	*Streptococcus* sp.			3–5,11,15,20
	S. salivarius	Oral commensal	No	10
	S. viridis	Oral commensal	No	11
	S. parasanguinis	Oral commensal	No	10
	S. bovis	Ruminal commensal	Yes	10
Lactobacillaceae	*Lactobacillus* sp.	Vaginal, oral, gut commensal, environment	Yes	3,11,15,16,22,23,25
Bacillaceae	*Bacillus* sp.			5,20
	B. cereus	Soil, gut commensal	Yes	4
Leukonostocaceae	*Weissella* sp.	Environment	Yes	3
Veillonellaceae	*Veillonella* sp.			6,15,17,22
	V. parvula	Oral commensal	No	10
	V. dispar	Oral commensal	No	24
	Megasphaera sp.	Vaginal commensal	No	10
Actinobacteria				
Bifidobacteriaceae	*Bifidobacterium* sp.			3,6,15,20,22,23,25,26
	B. bifidum	Major gut, vaginal commensal	No	10
	B. breve	Major gut, vaginal commensal	No	10
	B. longum	Major gut, vaginal commensal	No	10
	B. dentium	Oral commensal	No	10
Coriobacteriaceae	*Atopobium* sp.	Vaginal commensal	?	3
	A. parvulum	Vaginal commensal	Yes	10

Table 1 (*continued*)

Systematic groups	Species	Ecology	Potential opportunistic pathogen	References
Corynebacteriaceae	*Corynebacterium* sp.	Environment	No	5,16
Bacteroidetes				
Bacteroidaceae	*Bacteroides* sp.	Major gut commensal	No	11,15,16,22,23,25
	B. fragilis	Major gut commensal	No	4
	B. thetaiotaomicron	Gut commensal	No	24
	Parabacteroides sp.	Gut commensal	No	15
Fungi	*Candida* sp.	Environment	Yes	4,5,20

disease. In fact, frequently, the same species have also been found in physiologically matched healthy individuals.

Infectious agents associated with outbreaks of NEC: Data on hospital outbreaks of NEC provide valuable insight into the infectious nature of this disease. Such outbreaks have prompted intense scrutiny of the potential cause, which, in many cases, has resulted in tentative identification of the putative pathogen. NEC outbreaks have been described where the infectious agent was community-acquired rather than hospital-acquired.[38] Viral, bacterial, and fungal agents have been implicated in clinical NEC. Information on the likely agents causing the clinical outbreaks is summarized below.

Viral agents

Rotavirus: Rotavirus is often nosocomial and sometimes asymptomatic in neonates. The GI symptoms of rotavirus infection include diarrhea, bloody stools, abdominal distention, and intestinal necrosis.[39–44] Rotavirus enteritis may be associated with *pneumatosis intestinalis*, presumably due to the presence of clostridia.[45]

Norovirus: Norovirus has been associated with at least two cases of hospital outbreak of NEC. Like rotavirus, norovirus can be asymptomatic and affects only susceptible individuals.[46,47]

Echovirus 22: This rare virus has been implicated in one hospital outbreak of NEC. Out of 19 infected patients, 12 had diarrhea and 7 had symptoms of NEC.[48]

Parvovirus B19: A group of pre-term neonates developed NEC following transfusion of red blood cells contaminated with this virus.[49]

Bacterial agents

C. sakazakii: This food-borne pathogen has been implicated in clustered outbreaks of NEC. Strains isolated from patients were identical to strains isolated from tainted formula or milk, which could be interpreted as positive identification of the pathogen.[50,51] *C. sakazakii* is one of the few proven NEC pathogens. Studies with *C. sakazakii* provided important insights into the role of infectious agents in the pathogenesis of NEC (see below).

Clostridia: Species of clostridia are omnipresent and constitute part of the normal intestinal microbiota. These obligate anerobes survive in the environment due to their ability to form spores. Most clostridia are innocuous in a normal host, but may be pathogenic in compromised hosts such as pre-term infants. Alternatively, they may exacerbate the damage caused by other pathogens. Clostridia are believed to be responsible for *pneumatosis intestinalis* and portal vein gas during NEC. A few strains of clostridia have been associated with hospital outbreaks of NEC.[52–54]

Pseudomonas aeruginosa: These bacteria have been implicated in outbreak of sepsis and NEC in the NICU. Over 1/3 of sepsis cases caused by *P. aeruginosa* were associated with NEC.[55,56]

Klebsiella pneumoniae: Extended-spectrum β-lactamase-producing strains of *K. pneumoniae* have been reported to cause hospital outbreaks of sepsis associated with a high incidence of NEC.[57,58]

Acinetobacter: There has been a cluster of sepsis cases related to *Acinetobacter*. In five patients, the condition was also associated with NEC.[59]

Suspected, but unproven infectious agents. In many retrospective studies and individual cases, a number of suspected pathogens have been identified (Table 2).

Lessons from proven NEC pathogens

C. sakazakii

This species is an emerging pathogen that has been implicated in neonatal NEC, sepsis, and meningitis.[60] To demonstrate the ability of *C. sakazakii* to act as a pathogen in NEC, we used our well-established animal model, which employs formula feeding and hypoxia to induce NEC-like disease in newborn rats. Introduction of a human isolate of *C. sakazakii* (strain 51329), but not *E. coli*, at 10^5 cfu significantly increased the degree of intestinal inflammation, reported as NEC pathologic grade. The effect of *C. sakazakii* was dose-dependent: 10^3 cfu did not increase pathologic grade over baseline, whereas 10^7 cfu caused death in over 95% of

Table 2
Infectious agents suspected in clinical cases of NEC

Strain, species or group	References
Viruses	
Cytomegalovirus	72–79
Adenovirus	80
Coronavirus	81
Echovirus 7	82
Torovirus	83
Astrovirus	84,85
Bacteria	
Enterobacteriaceae	86,87
Enterobacter aerogenes, *E. cloacae*	88,89
Shigella sp., *S. boydii*	90,91
Salmonella enteridis	92
E. coli O157:H7	93
Klebsiella pneumoniae	88,94
Enterococcus sp., *E. faecalis*	94–96
Clostridium difficile	97
Staphylococcus epidermidis	98
Fungi	
Candida sp.	99,100
Absidia corymbifera	101,102
Mucor sp.	103–107

the animals before the end of the experiment. *C. sakazakii* did not invade enterocytes, but efficiently adhered to the epithelium *in vivo* and *in vitro*, causing enterocyte apoptosis and destruction of the villus tips.[61] Exposure to *C. sakazakii* stimulated the induction of the pro-inflammatory cytokine IL-6 in enterocytes. Importantly, *C. sakazakii* also increased nitric oxide levels by inducing iNOS, a hallmark of epithelial damage during NEC.[62] High levels of NO were responsible for the increased apoptosis.[63] *C. sakazakii* caused significantly more epithelial damage in *mdr1a*$^{-/-}$ mouse neonates than in wild-type congenic controls. Because *mdr1a* deficiency predisposes the epithelium to damage caused by intestinal bacteria, this result indicates that *C. sakazakii* displays increased virulence in a compromised host, and thus acts as an opportunistic pathogen.[64] The pathogenic effects of *C. sakazakii* 51329 depend on the outer membrane protein, OmpA, as mutants lacking this protein are non-pathogenic.[65] To characterize the ability of various isolates of *C. sakazakii* to cause epithelial damage, we screened a group of independently isolated strains for their ability to disrupt epithelial monolayers. Only 3 out of 24 strains induced barrier leakage, disrupted tight junctions, and induced enterocyte apoptosis. All of these three strains were of human origin.[66] Others have also reported strain-specific differences in adherence and epithelial damage properties in *C. sakazakii*.[67–70] Thus, *C. sakazakii* is a proven opportunistic NEC pathogen whose virulence is strain-specific. Koch's postulates have also been satisfied for clostridia and *K. pneumoniae*. In gnotobiotic quails, strains of *Clostridium butyricum*, *Clostridium perfringens*, and *Clostridium difficile* isolated from NEC patients caused cecal lesions similar to those observed in NEC; however, NEC did not occur if bifidobacteria were introduced along with clostridia.[71] Therefore the latter acted as opportunistic pathogens. A strain of *K. pneumoniae* caused NEC in neonatal mice. Development of NEC in this model was dependent on chemical obliteration of the host's Paneth cells, indicating that *K. pneumoniae* acted as an opportunistic pathogen. In summary, these studies provide experimental evidence for the concept of opportunistic pathogens as causative agents in NEC.

Conclusion and further directions

Microbiome studies in neonates have clearly established low complexity, high inter-individual diversity, and fluidity as key characteristics of early microbial populations of the gut. Correlating the patterns of intestinal microbial populations with NEC proved more difficult. Although several laboratories initially reported an association between NEC and specific groups of bacteria, such as non-*E. coli* Gram-negatives or clostridia, the majority of studies have failed to establish such an association. Clinical NEC has been associated with a number of viral, bacterial, and fungal species. In most cases, the role of specific microbes as putative NEC pathogens has not been conclusively established to fulfill Koch's postulates. In those cases where Koch's postulates have been met, it was demonstrated that causative agents of NEC act as opportunistic pathogens. Studies examining the pathogenicity of *C. sakazakii* demonstrated that the ability to cause epithelial damage in NEC models is not a property of bacterial species as a whole, but rather a characteristic of specific strains. Strain-specific virulence may explain why the same microbial species may or may not cause the disease. Further progress in understanding the role of microbes in the pathogenesis of NEC will require the identification of specific strains of opportunistic pathogens using animal and cell culture models. Detailed knowledge about a multitude of strains that can act as NEC pathogens will lead to better diagnostics and pathogen-tailored antibiotic therapies.

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