0031-3998/08/6302-0117
PEDIATRIC RESEARCH
Vol. 63, No. 2, 2008
Printed in U.S.A.

REVIEW ARTICLE

Understanding the Susceptibility of the Premature Infant to Necrotizing Enterocolitis (NEC)

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ABSTRACT: Necrotizing enterocolitis (NEC) is the most common life-threatening gastrointestinal emergency encountered in the neonatal intensive care unit. Despite advances in neonatal care, NEC remains a leading cause of morbidity and mortality among premature infants. Epidemiologic studies have identified multiple factors that increase an infant's risk for the development of NEC, although premature birth, bacterial colonization, and enteral feeding are thought to play central roles in disease pathogenesis. Appreciating factors that underlie the susceptibility of prematurely born infants to NEC is important for the development of new strategies aimed at the prevention and treatment of disease. In this review, we discuss defense mechanisms in the intestine and discuss how these systems may be insufficient in the prematurely born infant and thereby further contribute to initiation of NEC. In addition, based on a review of the literature, we suggest that, although numerous bacterial and viral pathogens have been associated with NEC, no individual organism is known to be responsible for disease. Finally, we comment on the possible role for probiotics in promoting maturation of intestinal defense mechanisms thereby attenuating or preventing the sequence of events that lead to NEC. **(*Pediatr Res* 63: 117–123, 2008)**

Necrotizing enterocolitis (NEC) is the most common surgical emergency affecting the gastrointestinal tract of infants in the neonatal intensive care unit (NICU). The incidence of NEC varies from 0.3 to 2.4 infants per 1000 live births, with nearly 70% of cases occurring in infants born at less than 36 wk of gestation. Although the national incidence of NEC varies, NEC affects 2–5% of all premature infants and accounts for up to 8% of all NICU admissions (1). The overall mortality for NEC ranges from 10% to 50% but approaches 100% for infants with the most severe form of the disease, characterized by full-thickness destruction of the intestine leading to intestinal perforation, peritonitis, bacterial invasion, and sepsis. The majority of these infants are extremely low birth weight infants whose disease requires surgical intervention (2). Despite optimal medical and surgical management of NEC, infants that recover from disease may still require prolonged hospitalization for related complications, such as intestinal obstruction from scarring, liver failure due to a prolonged requirement for total parenteral nutrition, short bowel syndrome with intestinal failure and associated nutritional deficiencies, and associated defects in growth and development. Hence, NEC is a significant and growing heath concern for prematurely born infants.

The events that lead to NEC in premature infants are multifactorial and complex and include a history of a complicated early neonatal course and poor intrauterine environment and perinatal transition. The only consistent epidemiologic risk factors for NEC are prematurity and a history of enteral feeding, which may include a rapid advancement in feeding or high osmotic strength formula feeding (2,3). Despite its predilection for premature infants, NEC has also been described in infants born at term. The onset of disease in these infants occurs within days of birth and is often associated with a history of hypoxia such as in cyanotic congenital heart disease or ischemia as single risk factors, suggesting that the pathogenic sequence leading to NEC in term infants may be distinct from that in the premature infant (3). Although recent evidence supports that susceptibility to NEC, in premature infants, is linked to gastrointestinal tract immaturity, the mechanisms whereby these factors incite or promote disease are poorly defined. Understanding the defense mechanisms in the premature intestine and their contribution to NEC susceptibility and pathogenesis is therefore of great importance. Elucidation of these mechanisms may allow for the development of strategies to target components that normally maintain integrity of the epithelial barrier, and to prevent the characteristic inflammatory cascade of NEC. This review will focus on defense mechanisms in the gastrointestinal tract of premature infants and highlight how the state of immaturity in the intestine may permit the initiation and propagation of inflammation and pathogenesis in NEC.

Received May 15, 2007; accepted September 13, 2007.

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Supported by National Institutes of Health/National Institute of Allergy and Infectious Diseases grant AI-49473 (HRF, JSU, and VC).

Abbreviations: APC, antigen-presenting cells; **LPS,** lipopolysaccharide; **NEC,** necrotizing enterocolitis; **PMN,** polymorphonuclear leukocytes; **TLR,** Toll-like receptor

INTESTINAL EPITHELIUM AND NONIMMUNOLOGIC DEFENSE MECHANISMS IN NEC

A primary function of the intestinal epithelium is to provide a physical barrier between the inside of the body and the luminal environment of the intestinal tract. Although epithelial cells lining the intestine provide a physical barrier, barrier function is incomplete in the absence of both nonimmunologic and immunologic components of the intestinal defense system. The nonimmunologic components include the mucus layer overlying epithelial cells, which is composed of glycoproteins called mucins, as well as several peptides such as trefoil factor, which are produced by goblet interspersed within epithelial layer (4). The degree of mucus production and its composition changes with postnatal age, in response to bacterial challenge, following colonization by commensal microbial flora, and during intestinal epithelial restitution following injury (5). Therefore, deficits in production or in the composition of intestinal mucus or in its associated peptides are hypothesized to contribute to the ability of bacteria to invade and participate in the induction and propagation of NEC (6). Tight junctions between epithelial cells maintain the semipermeable properties of epithelial cells, thereby limiting the passage of bacteria as well its permeability to a myriad of other luminal macromolecules, while promoting the transcytosis and absorption of macromolecules generated during the normal processes of digestion. Immaturity in the composition and function of tight junctions, through the interactions of occludins and claudins (7) most likely accounts for the increased permeability of the epithelial barrier in the newborn, a finding that is further exaggerated in the premature infant. Moreover, cytokines produced by epithelial cells and cells of the innate and adaptive immune system in response to bacteria and their products, such as lipopolysaccharide (LPS), may further disrupt the function of epithelial tight junctions, thereby promoting the translocation of bacteria and their products and other macromolecules (8). LPS, a known ligand of TLR4, has been shown to stimulate transcription and translation of several pro-inflammatory cytokines by epithelial cells, including tumor necrosis factor (TNF), IL-6, and IL-8, all of which independently promote the inflammatory cascade characteristic of NEC (9). Indeed, immature human epithelial cells secrete more IL-8 than did a mature human epithelial cell line (10). Additionally, inflammatory cytokines such as TNF-α, interferon (IFN)-γ, and IL-1β, produced by epithelial cells and cells of the innate and adaptive immune systems (see below), further contribute to epithelial barrier dysfunction through the up-regulation of inducible nitric oxide synthase (iNOS), local overproduction of nitric oxide (NO), and the generation of reactive nitrogen intermediate, peroxynitrite ($ONOO^-$) (11). $ONOO^-$ has been shown to be directly responsible for increases in epithelial cell apoptosis and death and results in the decreases in epithelial cell proliferation and migration characteristic of NEC.

Proper barrier function in the intestine also includes the action of nonimmunologic defense mechanisms, such as physical and chemical factors at the epithelial-luminal interface, including acid secretion by gastric enterochromaffin cells, bile salt production by the liver, and the release of proteolytic enzymes by the pancreas in concert with the regular peristaltic contractions along the length of the intestine. Gastric acidity provides a first-line defense against bacterial passage into the proximal intestine. The premature human infant's gastric pH is initially high and then lowers toward mature levels with increasing age, ultimately reaching pH of <4 (12). In full-term infants and adults, migrating motor complexes propagate as waves along the intestine; however, these complexes are not present until approximately 34 wk of gestation (13). Disordered peristalsis in premature infants may therefore contribute to stasis of intestinal luminal contents and bacterial overgrowth, particularly in concert with formula feeding. Thus, pro-inflammatory cytokines and mediators produced by epithelial cells in conjunction with cells of the innate and adaptive immune system compound baseline defects and immaturity in barrier function associated with the premature infant at birth, thereby promoting the inflammatory cascade characteristic of NEC.

INNATE AND ADAPTIVE IMMUNOLOGIC DEFENSE MECHANISMS AND NEC

The gastrointestinal tract is home to the largest lymphoid tissue in the body and is responsible for coordinating immunologic defense mechanisms between the adaptive and innate immune systems (14). The gut-associated lymphoid tissue (GALT) consists of cells of the adaptive immune system called lymphocytes, as well as cells of the innate immune system, including resident tissue macrophages, dendritic cells, specialized epithelial cells called M cells overlying the Peyer's patches, and Paneth cells located in the crypt region of the intestine.

Macrophages are well-recognized phagocytic cells of the gut. However, both macrophages and dendritic cells act as professional antigen-presenting cells (APC), directly sampling antigens from within the lumen of the bowel, as well as functioning in the uptake of antigens processed by the M cell epithelium overlying Peyer's patches. In addition, professional APC, M cells, and epithelial cells lining the gut function as nonprofessional APC, presenting antigens to resident lymphocytes (15). The majority of antigens sampled directly from the lumen must be processed before their presentation to the cells of the adaptive immune system (16). Antigen degradation, processing, and presentation are vital steps in the initiation of an immune response. The processing and presentation of antigens is less efficient in the newborn, thereby reducing the ability of the adaptive and innate immune system to detect and respond to pathogenic organisms. Paneth cells produce a variety of antibacterial substances, including defensins, lysozymes, secretory phospholipase A2, and lectins. Defensins are small cationic peptides with broad-spectrum antimicrobial actions (17,18). Paneth cell secretion is stimulated by bacteria or by components of bacterial cell walls, such as LPS and lipoteichoic acid (LTA) (19). In a study by Salzman *et al.* (20), expression of α-defensin was found at significantly lower levels during fetal life when compared with the term newborn and adult. Therefore, the decreased

production of antibacterial products by Paneth cells may predispose premature infants to bacterial overgrowth, thus allowing NEC to develop. Although polymorphonuclear leukocytes (PMN) are not regular inhabitants of the healthy intestine, an increased number of PMN may be detected in the intestinal epithelium early in NEC. PMN production by the bone marrow and function is impaired in the newborn, potentially contributing to intestinal bacterial overgrowth and invasion (21). Likewise, the inflammatory cascade induced by bacteria may include a reduced oxidative burst in the premature infant due to low levels of NADPH, thereby reducing the function of the innate immune system (22).

Lymphocytes, including B and T cells, are found dispersed throughout the intestinal wall and lymphoid aggregates. Intestinal intraepithelial lymphocytes are T cells that reside between enterocytes. Both subsets are found in the lamina propria, as well as in defined lymphoid structures, including Peyer's patches, small lymphoid aggregates, and mesenteric lymph nodes. Despite considerable development of human T and B cells during fetal life, complete maturation of the systemic and intestinal immune system occurs after birth and in response to colonization by commensal microbial flora (23). The adult human small intestine contains 200–300 Peyer's patches composed of germinal centers with distinct B and T cell zones. Although Peyer's patches are present in the newborn, they are reduced in number and size and lack germinal centers. Furthermore, the newborn lamina propria is largely devoid of immunoglobulin A (IgA)-secreting plasma cells. IgA, which is normally secreted into the mucus layer, is a potent inhibitor of bacterial and viral epithelial attachment. As human intestinal IgA production does not peak until 4 y of age, a relative deficiency of IgA contributes to the susceptibility of the newborn to infections at the mucosal surface (24). Additionally, T cells in the newborn are less responsive to antigenic stimulation and have a decreased proliferative response to a variety of mitogenic stimuli (25). Therefore, it is plausible that intestinal immune system of the premature infant (similar to the systemic immune system has a reduced capacity to control or respond to the overgrowth and invasion by pathogenic and commensal bacteria that is characteristic of infants with NEC.

BACTERIA AND THEIR PRODUCTS IN THE PATHOGENESIS OF NEC

Whether bacteria are primary in the initiation of NEC, or whether bacterial invasion occurs secondarily following the breakdown of the epithelial barrier is not known. The presence of pneumatosis intestinalis, air within the intestinal wall, however, indicates that bacterial fermentation at least accompanies disease. Thus far, however, a single bacterial species or virus has not been consistently isolated in cases of NEC (26–29). *Enterobacteriaceae* sp. are the most commonly described bacteria to be found in association with NEC (30–45). *Clostridia* sp. and *Staphylococcus* sp. have also been isolated from infants with NEC (37,46–57). Although bacteria are clearly the most commonly associated microbe with disease, isolation of viruses and fungus have been described (58–64) (Table 1). The data in Table 1 were derived from a PUBMED-based search of the English literature for NEC-associated pathogens using a modified Cochrane Collaboration data-quality scoring system. Due to the overall low quality of the data, however, a formal meta-analysis was not possible. The data are, however, consistent with the notion that NEC is unlikely to be a primary infectious or toxin-mediated disease. Although outbreaks of NEC have been described in association with feeding formula contaminated by *Enterobacter sakazakii* and breast milk containing *Staphylococcus* sp., it appears more likely that a dominant bacterial species gains access to the submucosa and circulation secondary to breakdown of the gut epithelial barrier, thereby playing a secondary but requisite role in disease.

Table 1. *Microorganisms reported in confirmed cases of NEC*

Pathogen*	Data class**	Total number of study patients	Number of articles cited
Enterobacteriaceae sp. (Includes *Escherichia, Salmonella, Klebsiella,* and *Enterobacter* species)	II–IV	3808	15 (30–45)
Clostridium sp.	III–IV	241	8 (46–53)
Staphylococcus sp.	II–III	1441	5 (37, 54–57)
Rotavirus	IV	54	3 (58–60)
Echovirus	IV	19	1 (61)
Coronavirus	II	91	1 (62)
Torovirus	III	521	1 (63)
Candida sp.	III	22	1 (64)
No prominent bacteria	II–III	211	4 (26–29)

* Results were analyzed according to the pathogen implicated using a modified Cochran collaboration class description to stratify the data.

** Class I: Prospective, randomized, blinded, controlled clinical trial with defined outcome measurements. Class II: Prospective matched group cohort studies in a study population that otherwise meets the criteria of class I data, or a randomized, controlled study that is deficient in one particular area, *e.g.* study power, poor definition of inclusion criteria. Class III: Controlled trials including well-defined natural history controls or patients serving as own controls in a representative population in which outcome assessment is independently determined by objective outcome measurement.Class IV: Uncontrolled studies, case series, or case reports.

Bacteria and their products have also been implicated in inflammatory bowel disease in humans (65) and in rodent models of inflammatory colitis and NEC (66–70). Detection and responses to microbial flora in the intestine occur through interactions with various pattern recognition receptors known as toll-like receptors (TLR) (71,72). These receptors recognize specific conserved pathogen-associated molecular patterns (PAMP), including glycoproteins, lipoproteins, glycolipids, peptidoglycans, fatty acids, and nucleic acids (73,74). Different TLR family members have been identified and are expressed in a variety of cell types including macrophages, dendritic cells, and enterocytes (73,75,76) (Table 2). TLR expression varies with postnatal maturation and cell type, indeed TLR deficiency has been demonstrated in macrophages and in the lung of neonatal mice (77). Differences in the expression of TLR may therefore alter a host's response to a commensal or pathogenic microorganism. For example, LPS is an outer membrane virulence factor associated with Gram-

Table 2. *Human TLR and their pathogen-associated ligands*

Receptor	Ligand
TLR1	Bacterial lipopeptides
TLR2	Bacterial cell wall lipoteichoic acid (LTA), lipoproteins
TLR3	Bacterial cell wall peptidoglycan, double-stranded RNA
TLR4	Bacterial LPS
TLR5	Bacterial flagellin
TLR6	Diacylated and triacylated bacterial lipopeptides
TLR7	Guanosine-rich and uridine-rich single-stranded viral RNA
TLR8	Guanosine-rich and uridine-rich single-stranded viral RNA
TLR9	Bacterial and viral DNA
TLR10	Unknown

Adapted from Abreu MT, Fukata M, Arditi M 2005 TLR signaling in the gut in health and disease. J Immunol 174:4453–4460.

negative bacteria and the ligand for TLR-4. *In vitro* studies have demonstrated that LPS may facilitate bacterial transcytosis in Caco-2 cells (78) and increase bacterial translocation (79). Although TLR ligands are expressed by several pathogenic species, commensal microbes also express these ligands. In fact, commensal signaling through TLR appear important for normal intestinal maturation and epithelial homeostasis (80). Hence, intestinal epithelial expression patterns of TLR-4 in the presence of altered intestinal flora may result in a variety of local responses, including altered bacterial translocation, gut homeostasis, and induction of cytokine pathways (71,81). Indeed, the administration of LPS to mice replicates the findings of human NEC (82,83). In mouse models of colitis and NEC, TLR-4 knockout mice have reduced inflammatory infiltrates when compared with wild-type mice (84). Thus, signaling *via* TLR by both enterocytes and cells of the innate and adaptive immune system may play a crucial role in triggering the inflammatory sequence in NEC.

BREAST MILK FEEDING AND PROBIOTICS IN PROTECTION FROM NEC

The microbial ligands recognized by TLR are not specific to pathogens, and signaling through TLR by commensal microbial flora in the gut may in fact enable normal assembly and function of the intestinal immune system (85,86). The intestine is devoid of bacterial flora at birth but is rapidly colonized by the recto-vaginal flora of the mother (55). Colonization by commensal bacteria is required for the normal development and maturation of the newborn intestine. Bacterial-host cross-talk modulates gut vascular development and promotes epithelial barrier function (87). For example, the intestinal flora stimulates the production of IgA in animal models (88). Indeed, the presence of IgA correlates with a decrease in bacterial translocation in the gut. In fact, the presence of *Bacteroides fragilis* in vaginally born infants, when compared with infants born by cesarean section, correlates with higher levels of IgA and IgM secreting plasma cells in peripheral blood (89).

The sequence of intestinal bacterial colonization after birth may provide protection from overgrowth by potential pathogens. The earliest bacterial species to colonize the intestine are facultative anaerobes including *Enterobacteriaceae* sp. and *Lactobacilli* sp., followed by anaerobic bacteria such as *Bifidobacterium* sp., *Bacteroides* sp., *Clostridium* sp., and *Eubacterium* sp. (90). *Lactobacillus* and *Bifidobacteria* sp. predominate by approximately 10-d post partum in the full-term breast milk–fed infant, whereas the microbial flora in the formula-fed infant is more heterogeneous, with only 50% of the level of *Bifidobacteria* sp. found in breast milk–fed infants. Significant differences in the microbial flora are seen in prematurely born infants hospitalized in the NICU, when compared with term infants in a non-NICU setting. Several factors contribute to the abnormal flora found in NICU infants, including the microbes endemic to a particular NICU and the frequent use of antibiotics. The intestine of hospitalized premature infants is likely to be colonized by pathogenic bacteria such as *Klebsiella* sp., *Enterobacter* sp., and *Clostridium* sp., with a marked paucity of *Bifidobacteria* sp. (91). Even in breast milk–fed very low birth weight infants it takes several weeks to approach the levels of *Bifidobacteria* sp. found in a full-term breast-fed infant (92,93). Premature infants fed human breast milk do, however, have a reduced incidence of NEC, when compared with formula-fed infants (94). A prospective multicenter study of preterm infants found an almost 10-fold increase in the incidence of NEC in formula-fed infants as compared with those who were fed breast milk (95). The positive effects of breast milk feeding may be due to a variety of antimicrobial products present in breast milk, including immunoglobulins, cytokines, oligosaccharides, lactoferrin, and glycoproteins with antiadhesive capacity for bacteria (96). In addition, the high levels of prebiotics, particularly in colostrums, such as fructo-oligosaccharides, may promote colonization of breast milk–fed infants with *Bifidobacteria* sp. thereby abrogating colonization by other pathogenic organisms (97). Moreover, lactoferrin is a milk protein found in high concentration in colostrum, which has been shown to decrease bacterial translocation from the gut and improve survival in LPS-fed animal models (98). Breast milk also contains cellular factors that likely facilitate the development and maturation of the immune system. For example, colostrum contains concentrated leukocytes, including macrophages (55–60%), neutrophils (30–40%), and lymphocytes (5–10%) (99). These cells are believed to be active and viable, and have been isolated from the feces of infants fed human breast milk.

The identification of probiotic bacterial species involved in gut homeostasis has stimulated interest in their use in the prevention and treatment of a variety of intestinal diseases. Although their mechanisms of action are poorly understood, it is believed that probiotic bacteria may impair growth of pathogenic species by stimulating production of nonfunctional receptor "decoys" in the mucosal lining, by promoting pathogen binding without internalization by epithelial cells, or the activation of an anti-inflammatory cascade (Fig. 1). Nonpathogenic enteric microbes may exact an immunosuppressive effect on epithelial cells through TLR interactions (100). The potential therapeutic benefit of probiotics has led to interest in their role in the prevention of NEC. *Lactobacillus* and *Bifidobacteria* sp. produce acidic end products during cellular metabolism, thereby lowering the pH of the intestinal microenvironment creating a locally unfavorable environment for pathogens. In mice, *Lactobacillus* sp. associate with Peyer's

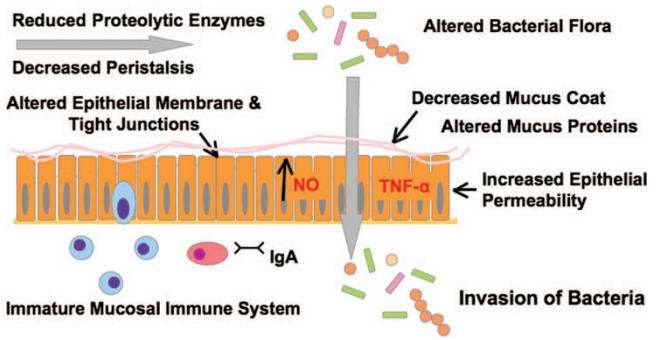

Figure 1. Immaturity of the intestinal epithelial barrier and neonatal mucosal immune system predispose the premature infant to bacterial infection/invasion triggering the pathogenic sequence in NEC. The immature intestinal barrier may lack several key protective mechanisms that normally prevents invasion by luminal bacterial flora. The compromised gut barrier together with an altered bacterial flora in premature infants may stimulate the production of pro-inflammatory cytokines further compromising intestinal defense mechanisms. The resulting imbalance between epithelial cell injury and repair leads to a vicious cycle of bacterial invasion, immune activation, uncontrolled inflammation, and gut barrier failure.

patches and exert effects on T-helper cells (101). Furthermore, it has been shown that *Lactobacillus* sp. binds to mucins and intestinal epithelial cells, and may be able to reverse the permeability of the immature gut (102). Additionally, it is hypothesized that *Lactobacillus* sp. may stimulate cytokine secretion and pathogen aggregation. In adults the administration of probiotic bacteria reduced the mucosal expression of several key pro-inflammatory cytokines including IL-1β, IFN-γ, and IL-8, suggesting that probiotics inhibit the production of pro-inflammatory cytokines (103) and in turn reduce epithelial cell apoptosis and the loss of epithelial barrier function. In addition, other studies demonstrate that *Lactobacillus* sp. increase the expression of intestinal mucins, which may directly enhance mucosal barrier function (104). Several randomized prospective studies in human infants suggest that oral probiotics decreased the incidence of NEC, without the development of other infectious complications such as sepsis (105–107). However, isolated case reports of sepsis following *Lactobacillus* sp. feeding have been described in both pediatric and adult populations following probiotic therapy (108,109). Hence, until further studies of efficacy and safety are available, routine use of probiotic therapy in premature newborns cannot be recommended (Fig. 1).

SUMMARY

Necrotizing enterocolitis accounts for significant morbidity and mortality in the premature newborn. The sequence of events leading to the development of NEC is complex and still incompletely defined. Well-developed models of disease and clinical studies will be required to understand why the premature newborn is susceptible to NEC. Our consensus supports that proposed by others: damage to the immature intestine after birth results in invasion of the intestine by bacteria. Bacteria, in turn, initiate a cascade of inflammation that leads to further destruction, or perforation of the intestine and to systemic infection. This may lead to the infant's death or to long-term morbidity in survivors that includes significant alterations in growth and development. Despite published reports over the last 30 years implicating many different microbial pathogens in the pathogenesis of NEC, no individual bacteria, fungus, or virus has been definitively shown to be causative of NEC. Although no single organism fulfills Koch's postulate, the role of microbes in the pathogenesis of NEC, similar to other inflammatory bowel diseases, is crucial. Probiotic therapy is an area of great potential, providing several strategies to improve epithelial barrier function perhaps through TLR signaling, precocious maturation of the mucosal immune system, or simple exclusion of pathogens from critical niches in the intestine. As our understanding of the microbial-immunologic underpinnings of NEC continue to progress, we believe that successful modalities in the prevention and treatment of NEC will emerge.

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