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REVIEW

Necrotizing enterocolitis: A multifactorial disease with no cure

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Abstract

Necrotizing enterocolitis is an inflammatory bowel disease of neonates with significant morbidity and mortality in preterm infants. Due to the multifactorial nature of the disease and limitations in disease models, early diagnosis remains challenging and the pathogenesis elusive. Although preterm birth, hypoxic-ischemic events, formula feeding, and abnormal bacteria colonization are established risk factors, the role of genetics and vasoactive/inflammatory mediators is unclear. Consequently, treatments do not target the specific underlying disease processes and are symptomatic and surgically invasive. Breast-feeding is the most effective preventative measure. Recent advances in the prevention of necrotizing enterocolitis have focused on bioactive nutrients and trophic factors in human milk. Development of new disease models including the aspect of prematurity that consistently predisposes neonates to the disease with multiple risk factors will improve our understanding of the pathogenesis and lead to discovery of innovative therapeutics.

Key words: Necrotizing enterocolitis; Diagnosis; Pathogenesis; Prevention; Disease models; Vasoactive/inflammatory mediators

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INTRODUCTION

Necrotizing enterocolitis (NEC) is an inflammatory bowel disease of neonates and remains one of the most common gastrointestinal emergencies in newborn infants[1]. Onset of NEC is often within the first three months of life and neonates who are of extremely low birth weight (< 1000 g) and under 28 wk gestation are the most susceptible[2]. Full term neonates account for 10% of all NEC cases while premature infants account for 90%[3]. With an incidence rate of 1%-5% for all newborns admitted to the NICU[1], a prevalence of 7%-14% of very low birth weight infants (VLBW, 500-1500 g)[4] and a mortality rate approaching 20%-50%[5], NEC continues to represent a significant clinical problem. In Canada, the incidence rate is 1.8 per 100 live births with a prevalence of 7% of VLBW infants[1]. Advances in obstetric and neonatal care have improved survival rates for smaller, more immature infants, and as more VLBW preterm infants survive the neonatal period, the population at risk for NEC increases[1]. No consistent association between sex, race, and rates of NEC has been identified. However, male VLBW infants and black infants are at greater risk of death[6]. Due to inadequate treatments and no effective preventative strategy, an estimated 20%-40% of babies with NEC require surgery[1] and 10%-30% experience significant morbidity including neurodevelopmental impairment, vision and hearing impairment, failure to thrive, feeding abnormalities, diarrhea, bowel obstruction, and short bowel syndrome[1,2,7]. The case fatality rate with surgical intervention is as high as 50%[1]. NEC is also a financial burden to the health care system with yearly hospital charges reported to be as high as $6.5 million in the US[8]. Thus, NEC continues to be an important health issue for preterm neonates.

DIAGNOSIS

Clinical signs and symptoms

The onset of NEC can occur suddenly within a few

Table 1 Bell's staging criteria for necrotizing enterocolitis

Stage	Systemic signs	Intestinal signs	Radiological signs
Ⅰ A (Suspected)	Temperature instability, apnea, bradycardia, lethargy	Poor feeding, emesis, ↑ pre-gavage residuals, mild abdominal distension	Normal or intestinal dilation, mild ileus
Ⅰ B (Suspected)	Same as above	Above and blood from rectum	Same as above
Ⅱ A (Proven)	Same as above	Above + absent bowel sounds + mild abdominal tenderness	Intestinal dilation, ileus, pneumatosis intestinalis
Ⅱ B (Proven)	Above + metabolic acidosis + thrombocytopenia	Above + definite abdominal tenderness	Above + portal vein gas + possible ascites
Ⅲ A (Advanced)	Above + hypotension, respiratory acidosis, neutropenia	Above + peritonitis, marked distension of abdomen	Above + definite ascites
Ⅲ B (Advanced)	Same as above	Same as above	Above + pneumoperitoneum

hours or may be preceded by several days of feeding intolerance[7]. Age at presentation is inversely related to gestational age at birth, with full-term infants often presenting in the first few days of life[1]. NEC affects the gastrointestinal tract and, in severe cases, may have profound systemic impact[9]. Initial symptoms may be subtle and can include feeding intolerance (gastric residuals, bilious vomiting), bloody diarrhea, temperature instability, lethargy, apnea, bradycardia, decreased peripheral perfusion, delayed gastric emptying, ileus, abdominal distension, or tenderness and respiratory stress[1,9,10]. Non-specific laboratory abnormalities can include neutropenia, thrombocytopenia, hyponatriemia, hyperglycemia, metabolic acidosis, and bacteria or infectious products isolated from blood, urine, or stool[9,11]. Serial C-reactive protein could be useful in the management of the disease. C-reactive protein distinguishes stage I NEC from ileus or benign pneumatosis and high levels predict development of complications such as strictures, abscess, or need for surgery[12]. Because early signs of this disease are non-specific, sepsis may be suspected before NEC[1].

Pathological findings

The ileum and proximal colon are the most commonly affected sites in NEC although any segment of the gastrointestinal tract can be involved including the stomach[13]. Severity of bowel wall necrosis ranges from a small localized mucosal necrosis of a bowel segment to transmural necrosis of the entire small intestine and colon in most severe cases[7]. In more advanced stages of NEC, pathological findings include gastrointestinal bleeding, inflammation, bacterial overgrowth, intestinal distension with multiple dilated loops of small bowel, pneumatosis intestinalis and portal air, intestinal perforation, coagulative necrosis, hypotension, septic shock, pneumoperitoneum, and intraabdominal fluid[2,10]. In 1978, Bell and colleagues[14] proposed a system for the uniform clinical staging of infants with NEC. They classified infants as having stage Ⅰ (suspect), stage Ⅱ (definite), or stage Ⅲ (advanced)[14]. Bell's staging criteria for NEC are guidelines for the management of NEC (Table 1).

Ideally, nutrition intervention would begin when an infant has one or more risk factors for developing NEC (i.e. preterm birth) or is at an early stage of disease.

Diagnostic methods

Early diagnosis of gut ischemia and mucosal inflammation/necrosis is crucial in the prevention of NEC or the progression of the illness to late stages requiring surgery and/or bowel resection. An abdominal radiograph and a chest x-ray are used to diagnose gastrointestinal tract abnormalities and changes in the size and shape of the lung and heart, respectively[10,11]. The experimental and clinical methods for early detection of gut ischemia or NEC include serum hexosaminidase, plasma amylin, serum cytosolic β-glucosidase activity, plasma pro- and anti-inflammatory cytokines, serum creatinine kinase isoenzymes, cerebro-splanchnic oxygenation ratio, GI tonometry, rectosigmoid pH monitoring, urinary EGF, D-lactate, or thromboxane, breath hydrogen, and MRI[8]. Most of these methods do not have high clinical utility either due to accessibility issues, high costs, and need for expert assistance or due to their poor properties as a diagnostic/screening test especially in the early stages of NEC. Some infants present so acutely and severely that morbidity or mortality cannot be avoided despite best treatment. Identification of a biological marker for early disease should allow more timely diagnosis and treatment, but no ideal marker has yet been identified. The serum of symptomatic infants tends to contain high concentrations of certain cytokines such as interleukin-8 (IL-8)[15]. Some studies suggest that serum concentrations of fatty acid binding protein in the intestine and liver (I-FABP and L-FABP) could also be used as markers for NEC[16,17]. L-FABP concentrations at the onset of clinical signs are highest in infants later diagnosed with stage Ⅰ NEC and I-FABP concentrations are highest in infants who later develop stage Ⅲ NEC[16,17]. More sensitive and accurate imaging studies, such as ultrasonography, could become helpful adjuncts to abdominal films in the diagnosis of NEC[18]. Further research is needed on new approaches for the medical management of NEC that might prevent disease progression and improved surgical outcomes to reduce complications such as short bowel syndrome.

PATHOGENESIS

Although the exact etiology and pathogenesis of NEC remains elusive, it is well established that NEC is a

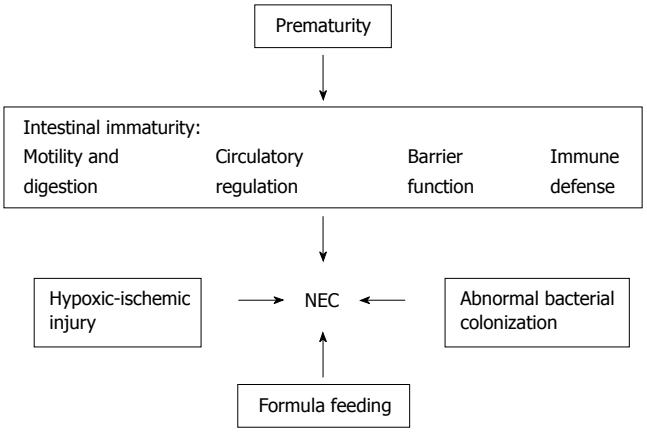

Figure 1 Pathophysiology of necrotizing enterocolitis (NEC).

complex, multi-factorial disease[2]. Besides pre-maturity, research suggests that other potential predisposing factors are hypoxic-ischemic injury, feeding with formula milk and colonization by pathological bacteria[1] (Figure 1).

Recent studies have shown that carrier state of genetic polymorphisms may be associated with perinatal morbidity, including NEC[19]. The hallmarks of NEC are loss of gastrointestinal motility, disruption of intestinal mucosal integrity, and mucosal inflammation, all of which result in the final common pathway, intestinal apoptosis and necrosis[4,20-23]. Several inflammatory and vasoactive mediators including platelet activating factor (PAF), cytokines, nitric oxide (NO), endothelin-1 (ET-1), prostaglandins, leukotrienes, and reactive oxygen species (ROS) are considered to play a synergistic and central role in the final inflammatory pathway leading to NEC[20]. The consequent breakdown of the mucosal barrier and impaired ability of the mucosa to heal leads to the self-perpetuating vicious cycle resulting in severe NEC, shock, sepsis, and sometimes death[8,24,25].

Prematurity

Prematurity is the only factor consistently found in epidemiological studies to be an independent determinant of NEC[2]. Up to 90% of infants with NEC are of low birth weight and the disease is more frequent and severe in those infants with the earliest post-conceptual age[7]. The increased susceptibility is attributed to an immature mucosal barrier and barrier response, changing intestinal microflora and increasing enteral volumes[2,23].

Immature intestinal motility, digestion, and barrier function

Intestinal motility is a critical factor in clearing antigens presented to the intestinal mucosal barrier from the gut lumen. The time available for absorption depends on the speed of luminal contents. Migratory motor complexes act as "house keepers" to propel luminal components caudally along the length of the small intestine. Immature intestinal motility and digestion may predispose preterm infants to NEC. Fetal studies in both animals and humans suggest that development of gastrointestinal motility begins in the second trimester, but matures in the third trimester[26-28]. Studies of intestinal motility have shown that premature infants can have immature motility patterns when compared with full-term infants and that maternal-fetal disease states that induce fetal hypoxia can further reduce postnatal intestinal motility[29-31]. Immature motility patterns alter normal peristaltic activity and result in overgrowth of anaerobic bacteria in the small intestine with malabsorption of dietary nutrients[23]. In addition, to impaired intestinal motility, premature infants have not yet developed the ability to digest and absorb nutrients and incompletely digested molecules could contribute to intestinal injury[32,33]. Lebenthal and Lee[34] showed that the function of the exocrine pancreas is limited in infants and that pancreatic insufficiency may last through the first year of life. Lack of stimulation of gastric acid and pancreaticobiliary secretions and their resulting proteolysis may adversely affect the intestine by allowing a greater bacterial and/or antigenic load. Thus, impaired digestion of nutrients, coupled with delayed transit time and bacterial overgrowth could result in intestinal injury with immature host and barrier defenses.

If structural or biochemical components of the intestinal epithelial barrier are not fully developed, bacteria may gain access to deeper tissues and cause inflammation. Intestinal epithelia are joined by tight junctions that regulate intestinal permeability and form by 10 wk gestation[35]. Studies show that intestinal permeability to macromolecules including immunoglobulins, proteins, and carbohydrates is highest in premature infants, particularly in those diagnosed with NEC[20,23]. When fully developed, the intestinal epithelial barrier can allow selective permeability to small ions, absorption of nutrients and control of bi-directional fluid flow. Enterocytes use chloride ions and water secretion to flush unwanted pathogens or toxins from the intestinal lumen. Fetal intestinal secretion and absorption are underdeveloped in preterm infants and mature gradually, under the influence of amniotic fluid, from 26 wk gestation to full-term[32]. Therefore, pathogens or toxins might not be efficiently washed from the intestinal lumen and could translocate across the leaky intestinal barrier in preterm infants.

Goblet cells are found throughout the small and large intestine. These specialized enterocytes secrete mucins, forming a thick protective layer over the intestinal mucosa. This mucus layer impedes direct microbial-epithelial binding and enhances removal of adherent bacteria[36]. Preterm infants have immature goblet cells. Developmental expression of mucin genes changes throughout the intestine and matches adult pattern expression between 23 and 27 wk gestation[37]. Microvilli of immature intestine also have altered glycosylation patterns[38]. Since carbohydrate sequences are recognition and attachment sites for microbes, changes in glycosylation patterns may influence the bacterial colonization pattern of the gut. An immature mucin layer might lead to increased intestinal permeability and enhanced bacterial adherence, potentially breaching the intestinal epithelial barrier and increasing susceptibility to injury.

Another aspect of the intestinal epithelial barrier that may not be functioning correctly in preterm infants is biochemical defenses. Paneth cells, which are specialized

secretory enterocytes located at the base of small intestinal crypts, secrete lysozyme, phospholipase A_2, and antimicrobial peptides (also secreted by absorptive enterocytes) that regulate composition and distribution of different bacterial populations[39,40]. Defensins (α and β) and cathelicidins are the two main families of antimicrobial peptides produced by intestinal cells[40]. These antimicrobial peptides have bioactivity against a wide range of microbes including bacteria, viruses, and fungi[41]. Some have a pro-inflammatory role and chloride secretory activity[42,43]. A better understanding of how biochemical defense molecules modulate host immune defenses *in vivo* should contribute to understanding the pathophysiology of NEC.

It is well established that growth factors, growth factor receptors, or their related signal transduction pathways are aberrant in the immature intestine. Epidermal growth factor (EGF) is a major trophic factor for the development of the intestine and the EGF receptor has been identified on the basolateral surface of enterocytes[44]. Exogenous infusion of EGF *in utero* has been shown to accelerate the maturation of intestinal enzyme activity as well as stimulate intestinal growth[45]. In the amniotic fluid, there is an increasing concentration of EGF as gestation progresses[46]. In fact, the salivary level of EGF is directly proportional to the gestational age of the infant[46]. Moreover, expression of EGF receptor involved in intestinal maturation and restitution is decreased in the preterm infant[7]. Recently, human data suggests a link between EGF production and NEC. Serum and salivary levels of EGF are significantly reduced in infants with surgical NEC[47]. Preliminary studies on the clinical use of EGF report improved epithelial regeneration with no significant toxicities[48].

It is unclear whether the intestinal epithelium of the infant can respond to injury to the same extent as the adult. In animals, infant intestinal epithelium turnover is much slower (4-5 d) than the adult (2 d)[49]. If the same finding holds true in humans, regeneration of injured mucosa in the infant will be much slower than the adult. Trefoil factor peptides (TFF1-3) are part of the protective mechanism operating in the intestinal mucosa and play a fundamental role in epithelial protection, repair, and restitution[50]. These secreted peptides have been identified in a site-specific pattern in the gastrointestinal mucosa and their expression has been shown to be up-regulated in early stages of mucosal repair[51,52]. The role of trefoil peptides in neonatal mucosal protection has not been well investigated. Intestinal trefoil factor is developmentally regulated and deficient in the premature neonate[20]. Recent studies demonstrated a lack of trefoil factor expression in response to NEC in the premature gut[53] and an insufficient proliferative response to reverse the mucosal insult observed in NEC[54]. Thus, impaired restitution of the mucosa may contribute to the cascade of bowel necrosis and generalized sepsis characteristic of NEC.

Immature intestinal immunity

Although the fetus at term may be sensitized to certain antigens, the fetus does lack a fully functional immune system and has a sterile gastrointestinal tract. Changes occur at, and soon after birth, in order that the immune system of the neonate becomes competent and functional and that the gut becomes colonized with bacteria. Exposure to bacteria during birth and from the mother's skin and the provision of immunological factors in breast milk are amongst the key events that promote maturation of the infant's gut and gut-associated immune system[55]. Dendritic cells play an important role in the initiation of the immune response. Microbial and antigenic-priming of dendritic cells develops different signals that drive the differentiation of naïve Th cells into Th1, Th2 or T regulatory cells[56]. Developmental changes in glycosylation patterns of immature dendritic cells may play an important role in development, maturation, and immune regulation[57].

Innate and adaptive immune defense systems are abnormal in developing neonates[20]. A possible mechanism for the pathophysiology of NEC is that reduced inflammatory signaling could allow bacterial overgrowth. Newborns are Th2 polarized and do not respond efficiently to IFN-γ[58]. Moreover, newborn macrophages exposed to LPS are defective in producing pro-inflammatory cytokines including tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), interleukin-6 (IL-6) and interleukin-12 (IL-12)[58,59]. Interestingly, inhibitory activity to toll receptors in neonatal but not adult plasma has been detected[60]. Neonatal monocyte and T cell production of the anti-inflammatory cytokines interleukin-10 (IL-10) and TGF-β are developmentally delayed[61]. Preterm infant polymorphonuclear (PMN) counts are lower and premature neonatal macrophages have reduced respiratory burst activation as compared with term newborns[62]. Under conditions of stress, PMNs of term and preterm infants do not function with normal phagocytic and microbicidal activities[63]. PMNs isolated from the blood of term and preterm neonates consistently display diminished chemotactic and adhesion capacities[64]. It is known that intestinal lymphocytes are decreased in neonates (B and T cells) and do not approach adult levels until 3-4 wk of life[20]. Newborns also have markedly reduced synthesis of secretory IgA and IgG in response to mitogens, reflecting decreased activity in the intestine[20]. Failure to activate inflammatory pathways in premature infants might prevent induction of anti-apoptotic, cytoprotective factors. Thus, developmental immaturity of the inflammatory response could increase susceptibility to apoptosis when cells are challenged by environmental stress.

Long-term survival requires inflammation as a defense mechanism, however, uncontrolled inflammation results in intestinal barrier damage, translocation of pathogens, further inflammation, and tissue damage. Some *in vitro* studies suggest that immature intestinal cells have a propensity for exaggerated inflammatory responses to pathogenic stimuli and researchers postulate that developmentally deficient expression of the NF-κB inhibitor IκB might allow greater NF-κβ activity[65,66]. NF-κB is a nuclear transcription factor that enhances the production of inflammatory mediators and is essential for innate immunity, adaptive immunity and cell survival[67]. In the human newborn, PAF-AH activity is decreased and

Table 2 Ischemic events associated with necrotizing enterocolitis

Perinatal asphyxia
Polycythaemia
Cyanotic congenital heart disease
Patent ductus arteriosis
Medications that ↓ superior mesenteric blood flow (cocaine)
Maternal pre-eclampsia

PAF synthesis pathways are increased. This imbalance places the newborn at special risk of an increased PAF response before adequate immune stimuli are developed[7].

Hypoxia-ischemia

Pathological findings of NEC associated with ischemic events (coagulative necrosis, Table 2) and the fact that NEC most commonly occurs in the distal ileum and proximal colon, which make up the watershed area of the superior and inferior mesenteric arteries, suggests that derangement of the circulatory system is involved[7].

Preterm neonates are more susceptible to hypoxia and intestinal ischemia because their system for regulating vascular resistance is poorly developed[68]. The most distinguishing feature of the newborn intestinal circulation is its very low vascular resistance due to substantial generation of endothelial derived NO when compared with ET-1[69]. Immature intestine handles the increased metabolic demands of growth by increasing blood flow and oxygen consumption[10]. However, during episodes of cardiovascular stress, infants are less able to raise intestinal blood flow and metabolic demands overwhelm the infant's ability to increase oxygen consumption[10]. Defective pressure flow autoregulation in response to hypotension occurs[68]. Consequently, hypoxia in tissues can occur. Hypoxia increases production of vasoconstrictor ET-1 and ischemia/reperfusion compromises production of endothelial derived vasodilator NO[69]. Thus, an imbalance between ET-1 and NO production by the newborn intestine following an initial ischemic insult might exacerbate existing intestinal ischemia. Whether the hypoxic/ischemic insult is primary, secondary or both an initiating factor and end result remains controversial. One plausible mechanism that is often cited is the "diving reflex", whereby blood flow is preferentially diverted to the heart and brain in preference to less vital organs[1]. Very early descriptions regarding the pathogenesis of NEC suggested a primary or early role for ischemia and hypothesized that a hypoxic/ischemic insult directly damaged the intestinal mucosa disrupting the neonatal gut barrier and promoting bacteria translocation and the inflammatory cascade[21]. Animal models suggest that NEC may not occur without significant reperfusion injury resulting from the generation of oxygen-free radicals at the restoration of blood flow and oxygen delivery after ischemia[9]. Inflammatory mediators may also cause intestinal ischemia by up-regulating ET-1 production and the expression of its receptor ET_A[69]. Current studies show a stronger association with prematurity, rapid feeding, abnormal intestinal colonization and inflammatory mediators than with ischemia[23]. Hypoxia-ischemia might contribute to the pathogenesis of NEC, but it likely plays more of a secondary role.

Formula feeding

Enteral feeds have a firm association with NEC as 90%-95% of NEC cases occur in infants with initiation/re-initiation of enteral feeds or recent volume advancement[2,20]. Infants receiving hyperosmolar formulas or rapid volume advancements are at greatest risk[20]. Although the mechanism is not well understood, enteral feeding has been reported to contribute to the development of NEC through disruption of mucosal integrity, blood flow and motility and through provision of a bacterial substrate[2,10]. Raising milk intake increases metabolic demands, making it difficult for the infant to expand mesenteric blood flow to meet demands[10]. As a result, intestinal hypoxemia may occur. Increased proliferation of potentially pathogenic bacteria may go on to invade the bowel wall[10]. Although the newborn gastrointestinal tract is sterile at birth, bacterial colonization occurs within hours[10]. Contact with the mother's vaginal flora begins this process, which is further developed by oral feedings and exposure to the environment[10]. In fact, breast fed infants are 10X less likely to develop NEC than formula fed infants, suggesting that breast milk contains multiple bioactive factors that influence host immunity, inflammation and mucosal protection. Breast milk notably increases the diversity of gastrointestinal bacterial colonization and contains immunomodulatory factors such as secretory immunoglobulin A, leukocytes, mucin, lysozyme, cytokines, lactoferrin, growth factors, enzymes, oligosaccharides, and polyunsaturated fatty acids not provided in commercially available neonatal formula preparations[20,55]. These factors are capable of inducing mucosal protection and neutralizing potent pro-inflammatory cytokines and phospholipids[55]. Glutamine and nucleotides may help in gastrointestinal cell metabolism[10]. EGF can directly improve gastrointestinal function and promote gut maturity[25].

Abnormal bacterial colonization and infection

The well-documented epidemics of NEC and the improvement in incidence and severity following the implementation of strict infection control measures validates the role of infection in the pathogenesis of NEC[2]. Furthermore, the regions of the intestine that are most often associated with NEC (ileum and proximal colon) have very high bacterial loads. Moreover, no cases of NEC have been described *in utero*, supporting the importance of bacteria colonization in the pathophysiology of NEC[20].

Although several bacterial and viral species have been associated with outbreaks of NEC (*Clostridium* sp, *Klebsiella* sp, *Staphylococcus epidermis*, *Escherichia coli, Rotavirus*), no single pathogen has been identified as causative and the ability of the microflora to colonize the epithelium and to ferment unabsorbed nutrients may be more important than the strain itself[13,70]. Recently, early abnormal colonization of stools with *Clostridium perfringens* has been correlated with later development of NEC[71]. *Clostridium perfringens* has

been isolated from 40% of infants with NEC, compared with 13% of controls[71]. Premature infants are especially susceptible to intestinal colonization by pathological bacteria due to their daily exposure to nosocomial flora and the likelihood of exposure to antibiotics and steroids on admission to NICUs[72].

Colonization of the gastrointestinal tract of the premature infant differs greatly from that of the healthy term infant[9,20]. Patterns of intestinal colonization also vary according to the type of enteral feeding[3]. The colonization of the hospitalized, premature infant gastrointestinal tract has less species diversity and fewer anaerobic species of *Lactobacillus* and *Bifidobacterium*[9,20]. Breast-fed infants have large amounts of protective, gram-positive *Bifidobacteria* in their intestine, contrasting with formula-fed neonates who are colonized predominantly by potentially pathogenic gram-negative *Enterobacteria*[3]. Gram-positive bacteria yield lactic acid during carbohydrate metabolism, which is readily absorbed from the intestinal lumen, whereas gram negative-bacteria ferment lactose into hydrogen, carbon dioxide and organic acids, producing distension, increased intraluminal pressure, decreased mucosal blood flow and pneumatosis intestinalis[3]. Enteral feeds and poor gastrointestinal motility associated with immaturity may promote stasis and bacterial overgrowth[3]. This microbial imbalance may represent a fertile environment for the pathologic overgrowth, binding and invasiveness of otherwise non-pathogenic intestinal bacterial species capable of triggering the inflammatory cascade with resultant NEC[9]. Recently, inappropriate immunologic responses of premature enterocytes to bacteria colonization have been implicated in the development of NEC[13]. Reports indicate that pathogenic stimuli including *Salmonella* and *E. coli*, produce exaggerated pro-inflammatory responses in immature intestinal epithelial cells[65,66].

Abnormal expression of pattern recognition receptors that recognize microbial signatures might also affect the way in which the intestine in premature infants responds to bacterial colonization. One of the first pro-inflammatory molecules to cross the intestinal barrier is lipopolysaccharide (LPS), which is a principal component of the outer cell wall of Gram-negative bacteria that recognizes and binds to toll like receptor 4 (TLR4)[21]. Circulating LPS is increased in patients with NEC, which inhibits epithelial restitution and initiates inflammatory signaling cascades within the enterocyte including activation of transcription factor NF-κB and expression of enzymes that produce apoptotic NO and pro-inflammatory eicosanoids and cytokines[21] (Figure 2).

In rats, intestinal epithelial cells up-regulate expression of TLR4 in response to stress-induced production of PAF, suggesting that up-regulation of TLR4 might explain how NEC develops in this animal model[73]. It remains unclear whether bacterial translocation into submucosa is a prerequisite for disease or whether the activation of the Toll-like receptors from endotoxin and other bacterial cell wall products is adequate to initiate the final common pathway of intestinal injury[20]. For premature infants at risk for NEC, there may be increased passage of bacteria

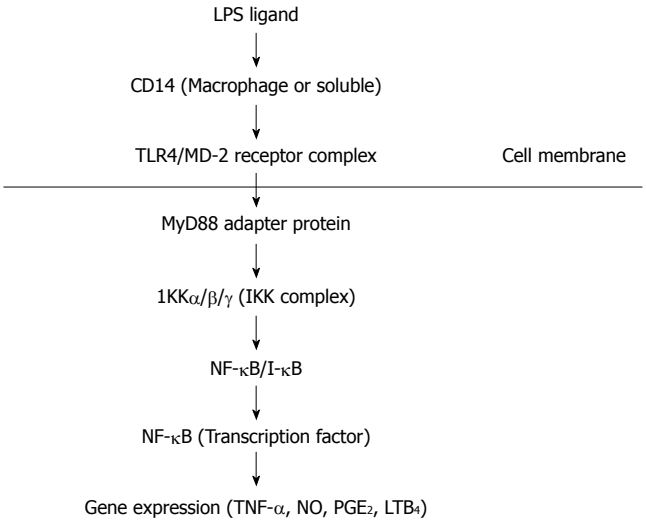

Figure 2 LPS-Induced signaling pathways leading to NF-κB activation.

from the gut into the systemic circulation and exaggerated pro-inflammatory responses[10]. Most of the defenses that would normally prevent passage of bacteria across the mucosal barrier-a well-functioning immune system, intact mechanical defenses and normal intestinal microflora are impaired in patients who are at risk for NEC[10]. Gram-negative bacteria translocate to regional lymph nodes and activate resident macrophages to release inflammatory mediators[2]. Bacteria endotoxins can leak into the systemic circulation causing release of inflammatory mediators, intestinal damage, shock and death[2,10].

Commensal bacteria interact symbiotically with the mammalian intestine to regulate the expression of genes important for barrier function, digestion, and angiogenesis[74]. Commensal bacteria can inhibit inflammatory pathways and perhaps contribute to the maintenance of homeostasis[75]. *In vitro* experiments show that a wide range of commensal bacteria can reduce inflammatory signaling in intestinal epithelia by inhibition of the NF-κB signaling pathway[76,77]. Preliminary work suggests that early colonization by probiotics (facultative anaerobes such as *Lactobacilli* and *Bifidobacteria*) reduces the risk of NEC in very low birth weight infants[78,79].

Genetics

Investigation of factors that might cause a genetic predisposition for NEC might eventually allow specific treatments or preventative strategies for the infants most at risk for this disease. Current technology allows for the detection and evaluation of genetic polymorphisms and their influence on disease development. Studies are now emerging which investigate the potential importance of specific polymorphisms for known NEC-associated inflammatory mediators. The presence of genetic variance may contribute to the inter-individual variance of cytokine response to inflammatory stimuli[19]. A family of intracytoplasmic pathogen recognition receptors has been shown to sense invading bacteria and activate gene transcription pathways that regulate immune and inflammatory responses. In a recent clinical study, VLBW

infants with mutations in a member of this family, NOD2, demonstrated increased susceptibility to bacterial sepsis[80]. Genetic polymorphisms of CD14, TLR4, and NOD2 are not associated with NEC in VLBW infants[81]. In preliminary studies, VLBWI with NEC were shown to be less likely to possess the interleukin-4 (IL-4) receptor α-chain mutant allele compared to infants without NEC[19]. The investigated variant of IL-4 receptor α gene is associated with enhanced transduction of IL-4 signals which shifts the development of lymphocytes to a more pronounced Th2 state[19]. It is speculated that the elevated number of Th2 cells in carriers of this genetic polymorphism is a protective factor against the development of NEC[19]. The risk of NEC has also associated with the frequency of the IL-18^{607} AA genotype. The frequency of the AA genotype is significantly higher in infants with stage 3 NEC compared to stages 1 and 2[19]. Thus, the presence of the AA genotype may adversely affect the outcome of NEC through altered IL-18 levels, a cytokine that induces IFN-γ and amplifies Th1 cytokine production and IL-8 accumulation[19]. Another possible genetic factor is the pro-inflammatory cytokine TNF-α. In animal models, pretreatment with anti-TNF-α reduces the incidence and severity of NEC[82,83]. Investigators have not reported a genetic link between TNF-α gene variants and the disease[84].

Vasoactive and inflammatory mediators

Bacterial colonization and enteral feeds coupled with damage to and loss of the integrity of the immature gastrointestinal mucosa trigger the final common pathway leading to the development of NEC[9]. Inflammatory mediators are responsible for protecting the body from invading organisms and play a vital role in the pathogenesis of NEC[3]. Inflammation can be initiated by a variety of factors including exposure to the bacterial cell wall product, endotoxin, and ischemia reperfusion[20]. The release of potent biologically active phospholipids, cytokines, products of arachidonic acid metabolism, vasoactive mediators, neurotransmitters, and reactive oxygen species from the immature and damaged gastrointestinal cells and inflammatory cells amplify the inflammatory response, leading to tissue damage and NEC[9]. Studies of animals and human cell lines suggest that the balance between pro-inflammatory and anti-inflammatory modulatory factors in premature infants is pro-inflammatory[9].

NO

NO is a short-lived, labile free radical gas that reacts with a variety of biologically active substances[85]. Such reactions result in both local and systemic effects that modulate the inflammatory response in a variety of tissues[21]. The synthesis of NO in biological systems is regulated by nitric oxide synthase (NOS), which catalyzes the oxidation of the amino acid L-arginine to release citrulline and nitric oxide[21]. Although diverse molecular reactions of NO have been identified in physiological and pathological systems, the fastest and most biologically relevant reaction of NO is with superoxide to produce the potent oxidant peroxynitrite[21]. Peroxynitrite is a key intermediate that is generated at inflammatory sites and is responsible for mediating tissue injury, in part, through lipid peroxidation[21]. Three isoforms of NOS exist: Neuronal (nNOS) and endothelial (eNOS), which are calcium/calmodulin dependent and constitutively expressed, releasing physiologically low concentrations of nitric oxide (pM) and the calcium independent inducible isoform (iNOS), which releases toxic concentrations of nitric oxide (nM) in response to infection and inflammatory stimuli[86]. All three isoforms are expressed in the gastrointestinal tract[21,86]. The constitutive forms are expressed by endothelial cells, enteric neurons, gastric epithelial cells, and enterocytes[86]. In the gastrointestinal tract, NO mediates inhibitory nerve-related relaxation of intestinal smooth muscle and plays a role in regulating gut mucosal blood flow, mucosal permeability, intestinal motility and mucosal protection[85,86]. Normal smooth muscle sphincteric function as well as coordinated peristalsis is dependent on the integrity of intrinsic nitric oxide neurons of the myenteric and submucosal networks throughout all regions of the gut wall[85]. NO also maintains intestinal microvascular integrity by inhibiting platelet aggregation and leukocyte adhesion[86]. Ontogenic variation in constitutive NOS activity has been observed in different animal species, in humans and in different organs[85]. By contrast, iNOS expression and activity within the intestinal epithelium is normally low, although it may be increased 15-fold after 4 h stimulation with LPS[21]. NO and peroxynitrite have anti-microbial properties and play important roles in host defense against pathogens[86]. However, sustained high levels of NO production promote bacteria translocation following insults such as endotoxemia and ischemia-reperfusion injury[86]. The induction of iNOS mRNA expression by inflammatory mediators has been seen in animal models of NEC and in intestinal resections from patients with NEC where the predominant source of iNOS activity was the enterocytes. Endothelial NOS function is compromised in human intestine resected for NEC[87]. Poorly coordinated production of NO by NOS isoforms occur during the early phase of the disease and are involved in altered intestinal blood flow, ischemic damage, disassembly of tight junction proteins, and impaired healing typically seen in NEC[13]. Research suggests that NO participates in the pathogenesis of NEC by directly damaging the enterocyte monolayer and by disrupting the ability of the mucosa to repair itself[21]. Extensive apoptosis has been shown in the enterocytes of the apical villi of infants with NEC and this correlates with the degree of nitrotyrosine immuno-staining, a marker of NO release and tissue reactivity[88]. Toxic concentrations of NO have also been shown to decrease enterocyte proliferation and inhibit enterocyte migration[21]. It is proposed that peroxynitrite interferes with EGF receptor signaling in enterocytes[89].

ET-1

ET-1, a potent vasoconstrictor agent, is produced at several sites within the intestine including vascular endothelial cells, submucosal stroma, and circularis muscularis layers of the gut wall[69]. Although constitutively produced,

ET-1 production is increased by a wide range of stimuli including reduced flow rate, hypoxia and inflammatory cytokines[69]. ET-1 generates a profound degree of ischemia that is sustained for hours because of a unique interaction between ET-1 and its receptor[69]. If not balanced by concomitant vasodilatory stimuli, ET-1-induced ischemia can generate hypoxia and tissue death[69]. ET-1 induces vasoconstriction by binding to ET_A receptors present within the newborn intestine and whose activation can generate intestinal tissue damage when excessive amounts of ET-1 are present[69]. Recently, ET-1 was demonstrated to be associated with NEC. It has recently been shown that the tissue concentration of ET-1 is greater in human preterm intestine that demonstrates histologic evidence of NEC[90]. Moreover, it has been demonstrated that arterioles harvested from intestine exhibiting histologic evidence of NEC exhibits vasoconstriction and that the vasoconstriction can be reversed by blocking ET_A receptors[90].

Serotonin

Serotonin is an intermediate product of tryptophan metabolism and is primarily synthesized and released by enterochromaffin cells of the intestine (90%) and enteric/brain neurons (10%) in response to calcium influx, physical mucosal stimulation, nutrients, hypoxia, and elevations in intraluminal pressure[91]. Levels of serotonin in the gastrointestinal tract are regulated by a serotonin uptake transporter, SERT, present in the mucosa and enteric nerves[92]. The major function of serotonin in the gastrointestinal tract is stimulation of bowel motility, epithelial secretion, and vasoconstriction through serotonin receptor binding[91]. Disruption of serotonin homeostasis and signaling is commonly seen in several gastrointestinal motility and inflammatory disorders including bowel obstruction and inflammatory bowel disease[93]. In inflammatory bowel disease, serotonin levels and enterochromaffin cell numbers are increased[93]. The inflamed intestinal tissue releases more serotonin, has a reduced capacity to remove serotonin and the serotonin receptors are desensitized[93]. Some cases of NEC have been associated with maternal use of paroxetine, a long-acting serotonin re-uptake inhibitor[94].

PAF

PAF, an endogenous phospholipid with powerful pro-inflammatory actions, is synthesized by neutrophils, macrophages, endothelial cells, and enterocytes in response to endotoxin and hypoxia[10]. PAF formation begins with the conversion of a phosphatidylcholine precursor to a biologically inactive intermediate, lysoPAF, under the influence of cytosolic phospholipase A_2[73]. Subsequent acetylation of lysoPAF at the n-2 position by acetyltransferase completes PAF synthesis[73]. PAF has a very short half life as it is rapidly degraded by PAF-acetylhydrolase[73]. In the human newborn, PAF synthesis pathways are increased and the activity of the PAF-degrading enzyme PAF-acetylhydrolase is decreased[7]. This imbalance places the newborn at special risk of an elevated PAF response before adequate immune stimuli are developed[7]. Formula does not contain PAF-AH like human milk, leaving susceptible neonates at greater risk for NEC. PAF exerts its effects by binding to PAF receptors present on most cells[73]. Interestingly, PAF receptors are most highly concentrated in the ileum, the region of the intestine where NEC is very prominent[24]. Down-stream signaling includes elevation of cytoplasmic free calcium and stimulation of protein kinase C, mitogen-activated protein kinase (MAPK), and NF-κB with production of inflammatory molecules including iNOS, TNF-α, ET-1, IL-1, IL-6, and IL-8[73]. PAF also activates pathways that result in caspase activation and apoptosis[73]. PAF is one of the most extensively studied mediators of intestinal injury and has been indicated as an important mediator in several animal models and human analyses of NEC[4]. PAF infusion causes intestinal necrosis in animals and PAF receptor antagonists prevent injury following hypoxia, endotoxin challenge and ischemia reperfusion injury[20]. Human patients with NEC show high levels of PAF and decreased levels of plasma PAF-AH with levels correlating with NEC severity[4,24]. In immature or mildly damaged mucosa, the close proximity of bacteria and intestinal epithelial cells aids transcellular permeation of PAF into the mucosa and local entry of bacteria[10]. Injection of LPS and bacterial invasion leads to increased production of platelet activating factor, release of secondary inflammatory mediators and further mesenteric ischemia and damage causing clinical NEC[10,24].

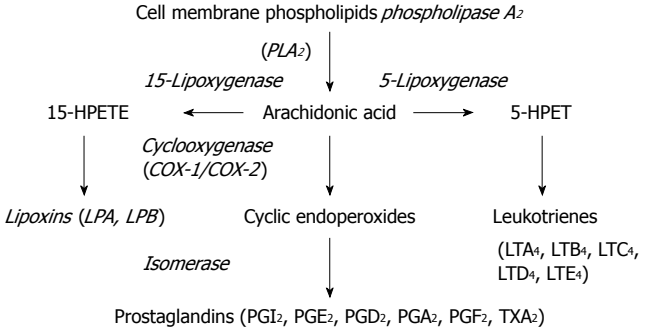

Figure 3 Metabolic pathways of arachidonic acid and eicosanoid production.

Eicosanoids

Arachidonic acid is a polyunsaturated fatty acid that is liberated from cell membrane phospholipids and serves as a precursor for many immune active lipids, collectively called eicosanoids (oxygenated C20 fatty acids)[95,96]. Classes of eicosanoids that signal in the immune system include prostaglandins, leukotrienes and lipoxins[95]. The major producers of eicosanoids are platelets, monocytes, macrophages, neutrophils, and mast cells, although with the exception of leukotrienes, they are also synthesized by a variety of non-immune cell types[95]. These lipid mediators are not stored in cells rather they are synthesized from arachidonic acid via three major metabolic pathways, either constitutively or in response to cell-specific trauma, stimuli, or signaling molecules[96](Figure 3).

The 15-lipoxygenase metabolic pathway results in the production of 15-hydroxyperoxy-eicosatetraenoic acid (15-HPETE) that serves as a precursor for the lipoxins

Table 3 Eicosanoid synthesis and actions

Eicosanoid	Cell/tissue origin	Target cell/tissue	Receptor	Action
PGE2	Most cells	Many cells	EP1-EP4	Fever, pain
PGI2	Endothelium	Platelet VSMC	IP	Declumping, vasodilation
PGF2	Uterus	Uterine SMC	FP	Contraction
PGD2	Mast cells	Lung Th2 cells	DP1/DP2	Asthma, chemotaxis
TXA2	Platelets	Platelet VSMC	TPα/TPβ	Aggregation, vasoconstriction
LTB4	Macrophage monocytes	Neutrophils	BLT1/BLT2	Promotes chemotaxis
LTC4/LTD4/LTE4	Macrophage monocytes	Lung SMC	BLT3/BLT4	Bronchoconstriction
LXA4	Leukocytes	Neutrophil	LXA4 R	Inhibits chemotaxis
LXB4	Leukocytes	NK cells	?	Inhibits cytotoxicity

LPA and LPB. Lipoxins exert anti-inflammatory activities through stimulation of macrophage phagocytosis of apoptotic neutrophils and inhibition of natural killer (NK) cell cytotoxicity and pro-inflammatory factor production[97,98].

Prostaglandins are end products of metabolism of arachidonic acid by constitutive and inducible cyclooxygenase isoforms (COX-1 and COX-2, respectively)[96]. The COX-1 enzyme accounts for basal prostaglandin synthesis for homeostatic regulation while COX-2 is involved in the synthesis of pro-inflammatory prostaglandins[96]. Leukotrienes are generated during the metabolism of arachidonic acid by the 5-lipoxygenase pathway and exert pro-inflammatory effects[95]. Prostaglandins and leukotrienes are emitted from their cell of origin and exert their effects in an autocrine or paracrine fashion by signaling through specific G-protein coupled receptors[95] (Table 3).

Cytokines

Pro-inflammatory cytokines are multifunctional proteins produced in response to inflammatory stimuli that communicate to the surrounding tissue the presence of infection or injury. Several pro-inflammatory cytokines that mediate inflammatory cell recruitment through activation and amplification of the immune response in local host defense have been implicated in NEC including TNF-α, IL-1β, IL-6, IL-8, IL-12, and IL-18[99-101]. Anti-inflammatory cytokines modulate the host's inflammatory response and if they fail to achieve their goal, pro-inflammatory mediators can continue, resulting in tissue injury[4]. The anti-inflammatory cytokines IL-4 and IL-10 have been implicated in NEC[4].

Granulocyte-Macrophage Colony Stimulating Factor (GM-CSF): The colony stimulating factors are a group of cytokines central to the hematopoiesis of blood cells, the modulation of their functional responses as well as the maintenance of homeostasis and overall immune competence[102]. GM-CSF is produced by a variety of cell types including macrophages, T lymphocytes, fibroblasts, endothelial cells, B lymphocytes, mast cells, eosinophils, and neutrophils[103]. In some cases, production of GM-CSF is constitutive as in a number of tumor cells lines; however, in most cases it requires stimulation of the producing cell with other cytokines, antigens, or inflammatory agents[103]. All of the biological effects of GM-CSF are mediated via the GM-CSF receptor which signals through MAPK and JAK/STAT pathways[103]. GM-CSF receptor expression is characterized by low number (20-200 per cell) and high affinity[103]. GM-CSF has pleiotropic and widespread effects on hematopoietic cells. It functions to promote the proliferation and maturation of neutrophils, eosinophils, and macrophages from bone marrow progenitors[103]. It also acts as a growth factor for erythroid and megakaryocyte progenitors in synergy with other cytokines[103]. The role of GM-CSF in cell survival results from apoptosis inhibitory mechanisms[103]. In addition to its role in the up-regulation of hematopoietic development, GM-CSF has been shown to have a profound influence on the biological functions of neutrophils, eosinophils, basophils, macrophages, lymphocytes, as well as endothelial cells[103]. These responses are widespread and point to a central role of GM-CSF in inflammation both through the direct activation of effector cells alone or in combination with other cytokines, as well as indirectly, through the stimulation of additional inflammatory mediator production[103]. Some of these biological effects include enhanced antigen presentation, chemotaxis, synthesis of a variety of soluble mediators and enzymes, release of reactive oxygen intermediates and histamines, antibody-dependent cell killing, and phagocytosis which contribute differentially to the immune defenses against bacterial, viral, fungal, and parasitic infections as well as tumor development[103]. Over-expression of GM-CSF leads to severe inflammation[104]. GM-CSF is used clinically to treat neutropenia in cancer patients undergoing chemotherapy, in AIDS patients during therapy and in patients after bone marrow transplant[103,105].

TNF-α: TNF-α release is triggered by a number of inflammatory stimuli including endotoxin (LPS), gram positive bacteria enterotoxin, viruses, fungi, and parasites[106]. Important cell sources of TNF-α in the gut are macrophages, lymphocytes, NK cells, neutrophils, endothelial cells, smooth muscle cells, intestinal epithelial cells, and enteric glia[100,106]. TNF-α exerts its effects by binding to TNF receptors[107]. Binding to the TNF receptor initiates local inflammatory responses through cell activation[107]. TNF-α is released early following injury and leads to a cytokine release cascade of IL-1β, IL-6, and IL-8[100]. It also inhibits release of glucocorticoids and the regulatory cytokines TGF-β and IL-10[106]. Some actions mediated by TNF-α include apoptosis induction, neutrophil activation, neutrophil recruitment, expression

of endothelium adhesion molecules, fever, and production and release of acute phase proteins, pro-inflammatory cytokines, NO, PGE2, matrix metalloproteases, PAF, and TXA_2[106]. The pro-inflammatory effects of TGF-α are mediated in part through NF-κB activation[106]. Elevated TNF-α has been detected in full thickness, resected bowel specimens of NEC intestine and in the plasma of babies with NEC[101]. In rat models of NEC, TNF-α induces hypotension, septic shock and severe intestinal necrosis synergistically with LPS[24]. Recently, a monoclonal anti-TNF-α antibody was demonstrated to reduce hepatic and ileal TNF-α production in a neonatal rat model of NEC[82]. Compared with other inflammatory bowel syndromes, TNF-α transcripts are lower in NEC[108]. Furthermore, studies indicate that the majority of TNF-α found in the gut lumen comes from Kupffer cells in the liver[99]. Taken together, these studies suggest that TNF-α plays a less significant role in the inflammatory cascade associated with NEC as compared with other intestinal inflammatory conditions.

IL-1β: IL-1β release is triggered by a variety of stimuli including microbial products, inflammation and TNF-α[100,109]. Important cell sources of IL-1β in the gut are macrophages, neutrophils, intestinal epithelial cells, endothelial cells, fibroblasts, dendritic cells, smooth muscle cells, and enteric glia[100,109]. IL-1β exerts its effects by binding to the IL-1 receptor and activating the transcription factor NF-κB[109]. Some actions mediated by IL-1β include macrophage activation, neutrophil recruitment, expression of endothelium adhesion molecules, fever, and production and release of acute phase proteins, IL-6, IL-8, and PGE_2[100,109]. Elevated IL-1β has been detected in full thickness specimens of NEC intestine[101]. Studies measuring plasma/serum IL-1β in NEC babies have not consistently reported elevated levels[15]. The difference in results may suggest that IL-1β is more predominant in the intestinal tissue in patients with NEC.

IL-6: IL-6 release is triggered by a variety of stimuli including microbes, microbial products, TNF-α, and IL-1β[100,110]. Important cell sources of IL-6 in the gut are macrophages, endothelial cells, and intestinal epithelial cells[110]. IL-6 exerts its effects by binding to the IL-6 receptor that signals through the STAT-4 pathway[110]. The IL-6 receptor is only expressed on hepatocytes and some leukocytes[110]. Some actions mediated by IL-6 include production of acute phase proteins, B cell growth, antibody production, T cell proliferation, and enhanced activity of hematopoietic growth factors such as GM-CSF[100,110]. Anti-inflammatory effects of IL-6 include production of tissue inhibitors of metalloproteinases and inhibition of superoxide production[111]. High levels of umbilical cord IL-6 have been associated with neonatal disease processes including NEC and systemic inflammatory response syndrome[112]. Elevated IL-6 has been reported in the plasma and stool of babies with NEC[4]. A study that looked at IL-6 mRNA expression in surgical intestine specimens from babies with NEC did not find a difference in comparison to control specimens[101]. Since IL-6 plays a dual role in inflammation it may serve as an anti-inflammatory mediator despite being correlated with increased morbidity and mortality in NEC patients.

IL-8: IL-8 synthesis and release is triggered in response to various stimuli including LPS, TNF-α, and IL-1β[113]. Important cell sources of IL-8 in the gut are macrophages, endothelial cells, intestinal epithelial cells, and fibroblasts[114]. IL-8 exerts its effects by binding to chemokine receptors CXCR1 and CXCR2 that signal through phospholipase c and PI3-kinase, respectively[113]. Some actions mediated by IL-8 are attraction of neutrophils and basophils to the site of inflammation, neutrophil activation and migration into tissues and production of acute phase proteins[114]. In intestinal specimens from patients with NEC, IL-8 mRNA expression was up-regulated, correlated with disease severity and was limited to areas with histological inflammation[4,115]. Similarly, plasma IL-8 levels are elevated in infants with NEC and levels correlate with clinical severity[4]. The vulnerability of the premature infant to develop NEC may, in part, be explained by the excessive inflammatory response shown by fetal enterocytes compared to more mature enterocytes[66]. When exposed to inflammatory stimuli, fetal intestinal cells exhibit a greater IL-8 response compared to mature intestinal cells[66]. This exaggerated response may partly be explained by the developmental down regulation of I-κB, an inhibitor of NF-κB[65].

IL-12: IL-12 synthesis and release is the early response to bacteria, bacterial products, and viruses[116]. Important cell sources of IL-12 in the gut are macrophages, neutrophils, B cells, and dendritic cells[116]. IL-12 exerts its effects by binding to IL-12 receptors present on T cells and NK cells[116]. Some actions mediated by IL-12 include IFN-γ production, Th1 and NK cell proliferation, cytotoxic T lymphocyte and Th1 cell differentiation, macrophage activation, and production of complement-fixing antibodies[116]. Several studies have identified putative NF-κB sites in the promoter regions of the IL-12 p40 genes[117]. IL-12 is a potentially important cytokine in the development of NEC. Halpern[99] localized IL-12 via immunohistochemistry to monocytes in the intestinal mucosa and lamina propria and correlated IL-12 positive cells with tissue damage in a neonatal rat model of NEC.

Interleukin-18 (IL-18): IL-18 is a cytokine that shares structural and functional properties with IL-1 and is pro-inflammatory inducing production of TNF-α and IL-1β[118]. IL-18 synthesis is triggered by LPS, Fas ligand and gram positive bacteria exotoxins[119]. Important cell sources of IL-18 in the gut are macrophages, dendritic cells, and intestinal epithelial cells[119]. IL-18 exerts its effect by binding to the IL-18 receptor present on macrophages, neutrophils, NK cells, endothelial cells, smooth muscle cells, and lymphocytes[119]. IL-12 upregulates the IL-18 receptor on lymphocytes[119]. Binding to the IL-18 receptor results in NF-κB activation. Some actions mediated by IL-18 include IFN-γ production,

enhanced NK cell cytotoxic activity, B cell antibody production, macrophage production of IL-8, activation and migration of neutrophils, phagocytosis, and integrin expression[119]. IL-18 can promote Th1 or Th2 lineage maturation depending on the underlying genetic influence and cytokine environment. The risk of NEC has been associated with the frequency of the IL-18^{607} AA genotype[19]. Recent data imply that IL-18, in the absence of IL-12, may facilitate the development of Th2 responses[118]. IL-18 is also essential to host defense against a variety of infections[119] and is potentially important in the development of NEC. Immunohistochemistry reveals the presence of IL-18 in intestinal epithelial and lamina propria cells which correlates with the degree of tissue damage in a neonatal rat NEC model[99]. Depending on the surrounding environment, IL-18 may play a destructive or protective role in NEC.

IL-4: IL-4 is a pleiotropic, immunoregulatory cytokine produced by Th2 cells, mast cells, B cells, and stroma cells[100,120]. IL-4 displays a wide variety of effects on B cell growth and differentiation, T cell growth and regulation, hematopoietic cells and differentiation of $CD4^+$ T cells into Th2 cells and is a key regulator in humoral and adaptive immunity[100,120]. IL-4 induces B cell class switching to IgE and upregulates MHC class Ⅱ production[100,120]. IL-4 is known to promote Th2 type responses and to exert immunosuppressive effects on macrophages including the suppression of pro-inflammatory cytokine production[100,120]. Although data are not available about the importance of IL-4 in NEC, isolated lamina propria mononuclear cells from the inflamed intestinal mucosa of children with chronic inflammatory bowel disease express and secrete IL-4 in lower amounts than control cells[121]. In preliminary studies, VLBWI with NEC were shown to be less likely to possess the IL-4 receptor α-chain mutant allele compared to infants without NEC[19]. The investigated variant of IL-4 receptor α gene is associated with enhanced transduction of IL-4 signals which shifts the development of lymphocytes to a more pronounced Th2 state[19]. It is speculated that the elevated number of Th2 cells in carriers of this genetic polymorphism is a protective factor against the development of NEC[19].

IL-10: IL-10 is the most important regulatory cytokine in the intestine and is primarily synthesized by Th2 cells, monocytes, and B cells[120]. Mononuclear production of anti-inflammatory mediators such as IL-10 is diminished in the newborn when compared to the adult, with preterm infants synthesizing less than term infants[122,123]. It is postulated that this phenomenon allows for persistent up-regulation of the inflammatory response and therefore increased susceptibility in the preterm neonate to long-term tissue damage after acute inflammatory conditions[124]. Interleukin-10 has been implicated as an inhibitor of pro-inflammatory cytokine production and of several accessory cell functions of the macrophage, T cell and natural killer (NK) cell lines[120]. Kuhn[125] demonstrated that IL-10 deficient knockout mice were predisposed to developing inflammatory colitis, suggesting that IL-10 works to counterbalance the response to enteric inflammatory stimuli. In fact, intraperitoneal IL-10 injections in a mouse model of ischemia/reperfusion injury reduced local and systemic inflammatory reactions[126]. Edelson[15] noticed significantly increased concentrations of IL-10 with severe NEC. IL-10 has also been shown to decrease the production of metalloproteinases[127] and suppress iNOS mRNA and NO expression in small bowel, liver and serum[128]. These findings indicate that IL-10 is a strong counter regulatory cytokine and that the potential of IL-10 to provide therapy in the setting of NEC is high. Perhaps the high levels of IL-10 in severe NEC are the body's response to dampen the inflammatory response.

ROS

One of the major endogenous sources of ROS in the intestine is the xanthine dehydrogenase/xanthine oxidase (XD/XO) system[129]. Xanthine dehydrogenase (XD), the precursor of XO, is constitutively and abundantly expressed in the intestinal villus epithelium, which catalyzes the conversion of hypoxanthine to xanthine, coupled with the reduction of NAD^+ to NADPH[130]. Because XO uses molecular oxygen rather than NAD^+ as an electron acceptor and thereby generates superoxide, XD to XO conversion (during ischemia) has been suggested to play the central role in intestinal reperfusion injury[129]. Following PAF challenge, it is the ileum that shows the most dramatic XD to XO conversion[130]. The central role of XO and ROS in causing the injury is supported by pre-treatment with allopurinol, a xanthine oxidase inhibitor, which largely prevents PAF-induced bowel necrosis[131]. Infusion of superoxide dismutase plus catalase also alleviates the injury[131]. In a piglet model of NEC, the level of the tissue antioxidant, α-tocopherol (vitamin E) was low in formula compared to colostrum fed piglets[13]. Thus, infants with NEC are under oxidative stress and may benefit from exogenous sources of antioxidants to replenish limited supplies.

NEC models

There are a number of accepted models used to study NEC and the cytokine cascade. These models serve to create necrotic bowel in animals to simulate that in the newborn child. LPS, PAF and TNF-α are often used to create intestinal ischemia. LPS is thought to mimic the bacterial overgrowth in the intestinal lumen and PAF and TNF-α cause a hypotensive response and shock[24].

Many animal models can simulate NEC, but often do not contain the aspect of prematurity that is seen in human NEC. The most physiological animal model that most closely resembles human NEC entails removing rat pups from the maternal uterus, exposing them to maternal milk, and stressing them with asphyxia, gram negative bacteria colonization, and artificial formula feedings[132]. After a few days of life, the rat pups begin to exhibit signs of NEC including intestinal distension and bloody diarrhea.

Other models have been described that do not physiologically resemble human NEC, but aid in the study of the disease process. These include inducing hypoxia for 5 min followed by 10 min with 100% oxygen[133], hypoxia for 50 s followed by cold exposure[134], superior

mysenteric artery clamping with or without PAF[135], intraarterial injection of TNF-α[136], and placing rats into a 100% nitrogen or 10% oxygen environment[24]. Finally, a rat model has been described by Chan[137] who created intestinal ischemia by increasing intraluminal pressure and injecting *E. coli* into the lumen.

In addition to *in vivo* animal models, various *in vitro* models have been created. The cell lines are often intestinal-derived and immortal such as CaCo-2, a human colon carcinoma cell line[138]. Inflammatory stimulants such as LPS and pro-inflammatory cytokines can be added to cell cultures which can then be analyzed to determine the presence or absence of specific cytokines. In addition, cells can be studied with regards to permeability, viability and expression of inflammatory markers after addition of certain stimulants or creation of hypoxic environments. Paracellular conductance can be assessed by measuring both trans-epithelial resistance (TER) and determining the rate of permeation of radiolabelled, hydrophilic probes between mucosa and serosa compartments of vertical diffusion chambers. It is unfortunate that primary cultures of human enterocytes have a limited life span (hours) in culture and therefore have not been useful as a model.

Symptomatic treatment and surgery

Due to the limited understanding of the fundamental biological processes that underlie the development of NEC, there is no cure for this devastating pediatric disease[21]. Symptomatic treatment of the infant with NEC begins with prompt recognition of the diagnosis and medical stabilization[2,9]. The treatment of NEC is based on the severity of the disease and is directed toward reduction of factors that aggravate the disease, treatment of infection and support of respiratory and cardiovascular systems[139]. Blood pressure should be closely monitored, all enteral feedings and medications should be discontinued and decompression of the gastrointestinal tract with placement of a gastric tube should proceed to evacuate residual air and fluid[9]. Rapid volume expansion with isotonic fluids may be necessary to reverse hypotension as well as frequent monitoring of blood glucose levels[9]. An intravenous infusion of total parenteral nutrition should begin during the 10-14 d bowel rest period[9]. The reinstitution of feedings generally is done in a slow and cautious manner, using an elemental formulation to allow for optimal absorption of all nutrients and to avoid further potential injury to the intestinal mucosa[7]. Broad-spectrum antibiotics including ampicillin and an aminoglycoside should be started as soon as cultures have been obtained[139]. With the increasing prevalence of infections from coagulase-negative staphylococcus, vancomycin may be used instead of ampicillin[2]. Anti-microbial choices should be guided by local resistance patterns[2,139]. Adjunctive therapy includes cardiovascular support (volume expansion with packed red blood cells), pulmonary support (oxygen and ventilation), and hematological support (blood product transfusion) as clinically indicated[1,139]. Indications for surgical intervention include peritoneal free air and signs of intestinal perforation[9]. Surgical intervention frequently results in resection of areas of necrotic bowel and exteriorization of viable ends (multiple ostomies) to allow for continued bowel decompression[2,9]. Recently, primary peritoneal drainage has been proposed as an alternative to surgical treatment. NEC STEPS and NET, prospective multi-centre randomized controlled trials, are currently underway to examine the effectiveness of primary peritoneal drainage versus laparotomy as primary therapy for perforated NEC in VLBW infants[140,141].

Table 4 Strategies to prevent necrotizing enterocolitis

Evidence-based support for efficacy	Limited data to support efficacy
Breast feeding	Cautious advancement of feedings
Trophic feeding	Fluid restriction
Antenatal steroids	Oral immunoglobulins
Enteral administration of antibiotics	L-arginine supplementation
	Polyunsaturated fatty acids
	Acidification of milk feeds
	Probiotics, prebiotics and postbiotics
	Growth factors and erythropoietin
	Free radical scavengers

PREVENTION

Strategies to prevent NEC fall into two major categories: Those with probable or proven efficacy and those that are experimental with unproven efficacy[2] (Table 4).

The most effective preventative strategies should improve both short-term and long-term outcomes for VLBW preterm infants and address the problems of prematurity.

Human milk

Human milk has been reported to reduce the incidence of NEC by up to 10 fold compared with infant formula whether using mother's own or donor milk[142]. Breast milk also reduces the severity of NEC[8]. The protective effect of breast milk has been correlated with its anti-inflammatory components (IL-10), growth factors (EGF), erythropoietin (Epo), lysozyme, immunoglobulins as well as pre- and probiotics that modulate intestinal microflora composition to the advantage of the host[55,143,144]. Research looking at a gut-stimulation, or gut-priming protocol has demonstrated potential benefits of promoting maturation of the gut by introducing early feedings with human milk[3]. The activity of acetyl hydrolase (PAF-AH), an enzyme that degrades PAF, is lower in neonates under 3 wk of age than at any other time[145,146]. The additional presence of PAF-AH activity may also partly explain the protective effect of breast milk, as infant formulas do not contain it[8]. Whether preterm human milk reduces the incidence of NEC is not clear at present[8]. Despite its advantages, it is important to appreciate that human milk alone will not eliminate NEC as cases are reported in neonates who have been breast-fed exclusively with human breast milk[8].

Trophic feeds

Initiation of trophic feeds, small volumes of breast milk

or formula, may overcome gut atrophy and inflammatory responses associated with prolonged bowel rest. Trophic feeds improve the activity of digestive enzymes, enhance the release of digestive hormones and increase intestinal blood flow and digestive motility in premature infants[147]. In addition, infants given early trophic feeds seem to have better feeding tolerance, improved growth, reduced period of hospitalization and decreased likelihood of sepsis compared with infants who are not[147]. Furthermore, early trophic feeds do not increase susceptibility to developing NEC. However, studies have not yet clearly delineated the best feeding strategies for premature infants[147].

Antenatal glucocorticoids

Antenatal glucocorticoid therapy has beneficial effects by suppressing inflammation and promoting gastrointestinal maturation and function including reduced mucosal uptake of macromolecules, decreased colonization with aerobic bacteria, reduced bacterial translocation, and increased activity of enzymes such as lactase, maltase, sucrase, and Na/K-ATPase[148,149]. A significant reduction in the incidence and risk of NEC following antenatal glucocorticoid therapy has been reported in several large, randomized control trials[150,151]. Mortality rate was also lower and there were fewer indications for surgical intervention[152]. Antenatal glucocorticoids have been reported to alter immune system development in very premature infants[153]. Mothers with the presence of infection or a condition that may compromise blood flow to the fetus (ex. pre-eclampsia) during pregnancy may be at risk of delivering a premature baby and may potentially benefit from early use of glucocorticoids. Thus, antenatal glucocorticoid therapy is a simple and effective strategy for global prevention of NEC and more research should be done to investigate potential impact on development.

Enteral antibiotics

Enteral antibiotics have been used as prophylaxis against NEC in low birth weight and preterm infants given the role of bacterial colonization in the pathogenesis of the illness. A systemic review and meta-analysis has reported that the administration of prophylactic enteral antibiotics resulted in significant reduction in NEC[154]. The trend towards a reduction in deaths was not significant[154]. The possible harmful effects of prophylactic antibiotics including the development of bacterial resistance and alteration of the natural microflora make it difficult to recommend this strategy for prevention of NEC.

Standardized feeding regimens (cautious advancement of feedings)

Inter-centre differences in clinical practice involving feeding regimens are significant factors linked to the prevalence of NEC in VLBW neonates[155]. A relationship between the rate of feeding advancement and an increased incidence of NEC exists[156]. A significant decline of 87% in the incidence of NEC and 29% in the risk of developing NEC was reported following implementation of a standardized feeding regimen in the form of clinical practice guidelines[157,158]. Parenteral nutrition coupled with minimum enteral feeding is the approach commonly advocated for the initial nutritional management of high risk infants and helps protect against NEC[159].

Fluid restriction

Excess fluid has been implicated in the pathogenesis of NEC. A systemic review and meta-analysis indicates that restricted water intake significantly increases postnatal weight loss and significantly reduces the risk of NEC[160]. Careful restriction of water intake (meeting the physiological needs without allowing significant dehydration) could be expected to decrease the risk of death from NEC without significantly increasing the risk of adverse consequences.

Probiotics

Since bacterial colonization can affect the course of many intestinal diseases, probiotics are emerging as a promising therapy. Probiotics are living microorganisms, which upon administration in sufficient numbers colonize the gut and exert health benefits beyond basic nutrition on the host[161]. As components of infant formula, typically used probiotic microorganisms are members of the genera *Lactobacillus*, *Bifidobacterium*, *Saccharomyces* and to a lesser extent *Streptococcus*. The beneficial effects of probiotics range from changes in intestinal permeability and enhanced mucosal IgA responses to an increased production of anti-inflammatory cytokines and protection of the mucosa against colonization from pathogens[162]. *Bifidobacteria* are the most common organisms recovered from the gastrointestinal tract of breast-fed neonates. Given the role of inappropriate gastrointestinal colonization by bacteria in the pathogenesis of NEC, probiotics may be beneficial in the prevention of NEC. Several studies have used different strains of probiotics and different administration regimens (length of treatment and dose) in preterm infants. None of the trials have reported adverse effects and no episodes of pathogenic infection caused by a probiotic organism have been observed[78,79,163,164]. Clinical trials show that probiotic supplements (*Lactobacillus acidophilus*, *Bifidobacterium infantis, Bifidobacterium bifidus,* and *Streptococcus thermophilus*) reduce the incidence and severity of NEC[78,79,165]. Larger clinical trials are necessary to confirm the safety and efficacy of this promising intervention to better define the benefits and risks for premature infants before wider use can be recommended.

Prebiotics

Another potential preventative strategy is to administer prebiotics, non-digestible dietary supplements, such as long chain carbohydrates or mucins, which promote proliferation of beneficial commensal bacteria[166]. Preliminary studies show increased *Bifidobacterium* stool colonization and decreased pathogenic bacterial colonization in preterm infants fed with formula containing prebiotics (90% short chain galacto-oligosaccharide, 10% long chain fructo-oligosaccharide) compared with infants fed control formula[167]. Furthermore, prebiotic treatment may have a positive effect on host immune function[168]. Because prebiotic supplements do not contain live microorganisms, they carry less risk of infection than

probiotic therapies. However, prebiotic administration has been associated with unwanted (but reversible) side effects such as flatulence, bloating and diarrhea[166].

Postbiotics

Another potential therapy involves bacterial metabolites or postbiotics, such as butyric acid, a short-chain fatty acid produced by commensal bacteria in the colon through anaerobic catabolism of complex carbohydrates. Butyrate is a major energy source for colonic enterocytes and has a widely recognized but poorly understood role in intestinal growth and differentiation[169,170], inflammatory suppression[171] and apoptosis[172]. Butyrate and other small molecule products might generate some of the beneficial effects of the normal flora (and exogenous probiotics and prebiotics), and could be a safe alternative therapeutic strategy. Butyrate has been administered with limited success in human inflammatory bowel disease[173], but there are as yet no studies in neonates.

Other products of commensal bacteria can induce protective responses that promote intestinal health. The beneficial effects of probiotic bacteria can be replicated by treatment with isolated microbe-associated molecular patterns (MAMPs)[174]. A MAMP is a molecular sequence or structure in any pathogen-derived molecule that is perceived via direct interaction with a host defense receptor[175]. For example, in mice unmethylated probiotic DNA ameliorates colitis[174]. Oral administration of inactivated probiotics (heat-killed commensals) or bioavailable toll-like receptor ligands could potentially induce beneficial TLR-mediated protective effects without carrying the infectious risk of probiotic therapies.

Arginine supplementation

Endothelial nitric oxide is an anti-inflammatory agent and vasodilator that is involved in the maintenance of intestinal vascular permeability, mucosal integrity and barrier function[21,86]. The plasma levels of the amino acid arginine, a substrate for NOS, have been shown to be low in neonates with NEC[176,177]. Arginine supplementation has recently been shown to reduce the incidence of all stages of NEC in a randomized, double blind, placebo controlled trial in preterm neonates[178]. Whether the beneficial effects of arginine supplementation in prevention of NEC are related to synthesis of glutamine or to its free radical scavenging action is currently unknown[179,180]. Guidelines have not been established for the safety and efficacy of L-arginine at doses above standard dietary practices in NEC[181].

Free radical scavengers (anti-oxidants)

Free radicals have been implicated in several neonatal disease processes including NEC[182]. A human recombinant superoxide dismutase is currently available and has been shown to prevent damage and attenuate eicosanoid release in a rabbit model of NEC[183,184]. The anti-oxidant vitamin E has been shown to reduce lipid peroxidation and intestinal lesions in a neonatal rat model of NEC induced by hypoxia-ischemia[183,184]. More studies on the therapeutic role of anti-oxidants in NEC should be done.

Acidification of gastric contents

Preterm neonates are often hypochlorhydria and enteric, Gram negative bacteria often colonize their stomachs, especially after gavage feeding[185]. Carrion and Egan[186] have documented that acidifying the feedings of preterm neonates to a pH low enough to inhibit gastric bacterial proliferation significantly lowers the risk and incidence of NEC.

Polyunsaturated fatty acids

Phosphotidylcholine (PC) is a major phospholipid constituent of mucosal membranes and the fatty acid component of PC, arachidonic acid, is a substrate for intestinal vasodilatory and cytoprotective eicosanoids[8]. Long chain polyunsaturated fatty acids (PUFA) have been proposed to modulate inflammation and immunity[187]. A clinical trial of formula feeds with or without supplementation with PUFA in the form of egg phospholipids in preterm neonates showed that the supplemented formula contained 7-fold more arachidonic acid and docosahexanoic acid and reduced the incidence of stage Ⅱ and Ⅲ NEC[188]. Recent evidence from an experimental study indicates that the protective effect of long chain PUFA is mediated by modulation of PAF metabolism and endotoxin translocation[189].

Oral immunoglobulins

A number of reports have been published, which suggest that oral immunoglobulins (IgA and IgG) have an immunoprotective effect on the gastrointestinal mucosa[190,191]. Premature infants have decreased levels of immunoglobulins, especially secretory IgA[192]. A reduction in the incidence of NEC following feeding an oral IgA-IgG preparation was reported as early as 1988[190]. A systemic review on oral immunoglobulin for the prevention of NEC did not show a significant reduction on the incidence of definite NEC[193]. No randomized controlled trials of oral immunoglobulins for the prevention of NEC have been carried out. Current evidence does not support the administration of oral immunoglobulin for the prevention of NEC.

EGF

EGF is a growth factor that exerts its effects by binding to the EGF receptor. EGF is an important constituent of gastrointestinal secretions and has multiple effects upon gut epithelial cells including cytoprotection, stimulatory effects on cell proliferation and migration, induction of mucosal enzyme and trefoil peptide expression, and inhibitory effects on gastric acid secretion[48]. Preterm neonates with NEC have diminished levels of salivary and serum EGF[194]. The presence of immunoreactive EGF receptors in gut epithelial cells of preterm neonates with NEC raises the possibility of using EGF for prophylaxis or treatment of NEC[195]. In a neonatal rat model of NEC, EGF treatment maintained intestinal integrity at the site of injury by accelerating goblet cell maturation and mucin production and normalizing expression of tight junction proteins[196]. Researchers have already warranted that the clinical use of EGF may be associated with a variety of problems and side

effects and that careful selection of patients and evaluation of risk-benefit ratios are necessary[197]. Given the potential for adverse effects and the fact that maturity alone is not a protective factor for NEC the use of any growth factors in preterm neonates warrants extreme caution.

Epo

The presence of Epo in human milk and the expression of Epo receptors on intestinal villous enterocytes of neonates suggest that Epo has a role in growth and development of the gastrointestinal tract[198-200]. Ledbetter[200] administered recombinant Epo for the prevention and treatment of the anemia of prematurity and demonstrated that the rEpo group had a lower incidence of NEC. Akisu[133,201] indicated that rEpo decreased lipid peroxidation but not PAF generation. Although not completely absorbed, Epo acts as a trophic factor in developing rat bowel whether given enterally or parenterally[199]. Current evidence indicates that the protective effect of rEpo may be related to inhibition of NO formation[202].

CONCLUSION

A variety of other experimental agents have been studied in search for an effective agent for the prevention of NEC. These include anti-TNF-α[82], PAF receptor antagonists[203], heparin-binding EGF-like growth factor[204], anti-inflammatory cytokines (IL-10)[205], pentoxifylline[206], intestinal trefoil factor 3[207], and glucagon-like peptide 2. Recent research has identified that complex glycosphingolipids in the form of gangliosides act as bioactive factors down-regulating many acute pro-inflammatory signals in the intestinal mucosa. Perhaps the solution to NEC will involve identification of an intestinal control mechanism that optimizes (or disregulates) the balance between pathways that signal inflammation, hypoxia, and mucosal cell growth or metabolism.

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