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PROBIOTICS AND NECROTIZING ENTEROCOLITIS

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INTRODUCTION

One of the most controversial areas in neonatology over the past few years is whether probiotics should be provided routinely to preterm infants for the prevention of necrotizing enterocolitis (NEC). The goals of this review are to:1) provide the reader with a brief overview of NEC and current concepts of its pathophysiology including the role of intestinal microbes, 2) discuss the microbial ecology of the intestine in preterm infants and factors that may lead to an unhealthy microbial intestinal environment (a “dysbiosis”), 3) summarize studies of probiotics in preterm infants, 4) elaborate on the need for regulation in this area, and 5) discuss alternatives to probiotics and what is the future for the prevention of NEC?

NECROTIZING ENTEROCOLITIS: More than One Disease

In this section, several aspects of developmental gastroenterology will be described as they relate to increased susceptibility to intestinal injury such as that seen in NEC. I will provide a brief description of NEC, the most fulminant gastrointestinal disease seen in neonatal intensive care. A more comprehensive review of NEC can be found in several reviews. [1–3] Although NEC can present in several ways, one frequent characteristic is a subtle onset presenting as a slightly distended abdomen, non-specific instability such as apneas or bradycardias, and changes in appearance and activity of the infant. These highly non-specific signs and symptoms may subside, but occasionally will fulminate to severe intestinal necrosis with systemic inflammation and shock. Mortality ranges between 20 to 30%, with a greater association in the least mature infants, but the diagnosis of NEC conferring a much greater relative risk of mortality to the larger infants because their baseline mortality is lower.[4] Significant morbidities include severe neurodevelopmental delays, shortened intestine and inflammatory processes that can affect other organs such as the liver with severe cholestasis. [5] It is thus a very expensive disease, not only in terms of its financial impact[6], but also in terms of long term physical disabilities and neurodevelopmental delays.

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Progress in the treatment and prevention of NEC over the past several decades has been almost nil.[7] Attempts to decrease incidence have included prolonged periods of nulla per os (NPO) wherein preterm infants would not receive food by the enteral route for weeks after birth or extremely slow institution of enteral feedings,[8] but subsequent studies suggested that this was counterproductive.[9,10] Studies in animals show that lack of enteral nutrition may lead to mucosal atrophy, decreased motility, decreased trophic hormones, and increased inflammation. [11] Numerous studies have now shown that providing at least small amounts of enteral feeding, especially human milk from early on after birth does not increase the incidence of NEC and may reduce the risk of other complications such as sepsis.[12,13]

Increased survival of very small infants who have a greater propensity to develop this disease than larger infants may be a partial reason for the lack of progress. Use of experimental animal models that do not directly reflect the highly multifactorial pathophysiology of this disease as seen in preterm infants, is also a likely reason for lack of progress. For example, a recent study from Sweden showed an increase in NEC together with decreasing mortality between the years 1987 to 2009. [14] Likewise, what we have been recording in our databases as “NEC” consists of a variety of entities, some of which may not even involve a necrotic intestine or primary inflammatory process. Hence, aiming a “magic bullet” directed at a poorly delineated disease process is likely to miss the target.

For example, babies with congenital left sided cardiac lesions such as hypoplastic left ventricle, interrupted aortic arch, coarctation of the aorta or even a severe left to right shunt due to a persistently patent ductus arteriosus are at increased risk to develop bowel ischemia, which does not involve a primary inflammatory process seen in typical NEC. Designing a preventative or therapeutic approach based on prevention of inflammation by altering the microbial environment in a disease that involves primarily lack of intestinal blood flow does not represent a reasonable approach for these forms of ischemic intestinal necrosis. Another entity, spontaneous intestinal perforation (SIP), may present with signs and symptoms similar to NEC, but involves minimal inflammation or necrotic intestine.[15] It occurs early after birth often without the infant being enterally fed. However, the radiologic presentation may be similar to NEC (free intraperitoneal air) and the therapy often includes peritoneal drainage without direct surgical inspection of the bowel and definitive diagnosis of NEC or SIP not being differentiated. Thus, SIP, sometimes mistakenly called “NEC” is unlikely to be amenable to therapies or preventative measures that include manipulations of the inflammatory response, nutritional composition or the intestinal microbial environment, and should not be clustered in a database with the diagnosis of “NEC”, because it can be misleading.

Another often unappreciated fact is that NEC may be very difficult to diagnose. The Bells Staging criteria[16] are often unhelpful in this regard. “Stage 1 NEC” is highly non-specific in that there are no criteria that provide for a definitive diagnosis. A distended abdomen, increased gastric residuals prior to the next feeding and non-specific signs such as increased apnea and bradycardia, all used in the diagnosis of “Stage 1 NEC” do not signify that the bowel is necrotic. Hence “Stage 1 NEC”, often used to name this set of signs and symptoms is a poor term that when used for inclusion of subjects in studies of NEC can only be misleading. For “Stage 2 NEC”, the diagnosis relies on radiographic criteria such as

pneumatosis intestinalis and portal venous gas. However, in many instances, this can also be misleading. Presented with the same radiograph, there is often disagreement among neonatologists, surgeons and radiologists as to whether some of the “gas bubbles” seen in the intestinal radiographs represent intramural air or feces. Often babies with free intraperitoneal air on radiograph are treated with a peritoneal drain and thus differentiation between NEC and SIP cannot be made because the bowel is not directly visualized on laparotomy. These difficulties in diagnosis provide a major challenge to design of clinical trials of NEC.

A disposition to NEC relates to several immaturities of the gastrointestinal tract. These include poor barrier function leading to increased permeability[17] with an activation of the highly immunoreactive cells that underlie the epithelial surface. Other aspects include a highly immuno-responsive potential of the toll like receptor (TLR) pathways especially TLR4,[18] which can be found on several intestinal cell types including the surface epithelium. Interaction of these receptors with microbial components is related to the intestinal inflammatory response, but the precise mechanism requires further elucidation.

Microbial dysbiosis (inappropriate colonization) has long been suspected to play a crucial role in the development of NEC.[19] Exploration of the microbial environment has recently undergone intensified scrutiny, largely because of newly developed technologies for microbial identification and funding engendered by the Human Microbiome Project. Several studies suggest that there is a progression of microbial taxonomy that differs in NEC patients versus controls prior to the onset of NEC. [20–22] This includes a high proportion of Proteobacteria in comparison to Firmicutes and Bacteroidetes at the Phylum level. Other bacteria taxa such as Klebsiella have also been seen strongly associated with NEC using 16S sequencing techniques.[22] Current studies are focusing on a more specific delineation of the functional aspects of the microbial milieu (metabolomics, transcriptomics, microbe-host interactions) that may led to NEC.

There are several other aspects of development of the intestinal tract which include the microvasculature and adaptive immune system which may at least be partially responsible for the interesting finding that many of these babies do not develop the disease until several weeks after birth, with a propensity to develop the disease between 28–31 weeks postmenstrual age. [23]

Other developmental components of the GI tract that may predispose to NEC that involve intestinal microbiota include a low basal output of gastrointestinal acid from the stomach. [24] This is of considerable interest since the studies show a correlation between the use of histamine receptor 2 (H2) antagonists and NEC or sepsis in preterm infants.[25,26] The mechanisms underlying this are poorly understood but studies suggest that Proteobacteria do not survive well in an acid environment and do better in a more basic milieu.[27] This fits well with the recent findings that Proteobacteria are present in high quantities relative to the Firmicutes phyla prior to the onset of NEC in preterm infants.[21,22] The Proteobacteria phylum contains many of the pathogenic microbes including E. coli and Klebsiella. Such studies suggest that a targeted microbial bacteriotherapeutic approach that engineers the gut microbiota toward a “healthy” composition might be feasible. [28]

Currently there is no treatment for NEC and there have been only minor changes in our treatment and preventative modalities in the past 40 years. It is clear that the best path toward eliminating this disease will be through interventions that interfere with the most proximal components of the pathophysiologic cascade. This would include dietary, microbial and other environmental manipulations. The rapid progression of the disease likely precludes interventions such as anti-cytokine therapy that might be given once the disease is already suspected.

MICROBIAL ECOLOGY OF THE INTESTINE

Since the beginning of the Human Microbiome Project in 2007,[29] studies that previously relied on cultivation of microbes are being rapidly augmented with several non-culture based techniques including quantitative PCR, microarrays, 16SrRNA and whole genome based studies. The conundrum termed the “great plate count anomaly” [30] (being able to see microbes in various settings without being able to culture them) is being explained by studies that rely on novel DNA based sequencing technologies and bioinformatic techniques to identify and characterize the function of non-cultivatable taxa. These taxa constitute the majority of microbes in the intestine. The promise that the new knowledge of the human microbiome holds has prompted some to use the term “our second genome”[31] for the genes comprising the microbiome in our bodies.

It is important for the reader to recognize that simply being able to name the taxa present in a certain niche before or during development of a disease process falls far short of being able to explain mechanisms of pathophysiology. Understanding how microbes interact with one another, what bioactive products they produce, and the interaction of these products with the host under various environmental (e.g., nutrition, antibiotic) conditions is critical. For example, samples of intestinal contents can now be analyzed using nuclear magnetic resonance and or mass spectrometry for small molecules such as the short chain fatty acids acetate, propionate and butyrate. Of these, butyrate is of special interest because of its strong relationship to the energy metabolism in the colon, its capability to induce proliferation, differentiation, formation interepithelial tight junctions and inhibition of histone deacetylase with resultant epigenetic potential.[32,33]

There are numerous factors that can affect the intestinal microbiota and lead to a dysbiosis. Of these factors, antibiotics are thought to play a major role and have been implicated to contribute to the development or exacerbation of various disease entities including NEC. [34,35] It can be argued that this association may not be causal because infants who are more ill initially may have both a higher incidence of NEC and the need for antibiotic use. However, several studies in animals are providing support for causality. Rakoff-Nahoum showed that obliteration of the microbial flora with broad spectrum antibiotics resulted in a greater propensity to intestinal injury and lower capability to induce repair. Interestingly, this impact upon propensity to injury and capability to induce repair is similar to that seen in genetically modified animals lacking TLR 4, TLR 2 and the signaling molecule MyD88. [36] Several additional studies show the effects of antibiotics on alteration of transcriptional regulation mechanisms in the intestine of newborn animals, as well as their long term effects

on the microbiome and how these effects lead to increases susceptibility to injury either by chemical agents or other microbes including viral infections.[37–39]

Nutritional composition may also be of major importance. Human milk, especially that provided by the baby's own mother, appears to play a major protective role in the prevention of NEC.[40] Numerous factors in human milk have been implicated in this protective role including bioactive molecules such as lactoferrin, oligosaccharides, long chain omega-3 fatty acids, and live immunocompetent cells. [41] More recently, the use of non-culture based technologies have explored the microbial milieu of human milk and have found that this may be a source of microbes for the infant.[42] The hypothetical source of these microbes is reviewed elsewhere,[43] but one intriguing finding is that the microbes in the human milk from one mother appear to differ little over a period of several months, but the microbes in the milk from one mother differ significantly from the microbes of another mother, suggesting a mother-specific role for these microbes for each individual mothers' infant.[42]

Other factors are likely to also play major roles in the development of the microbial ecology of the preterm infants gastrointestinal tract. These include mode of delivery, postnatal bathing, the use of incubators versus radiant warmers, the frequency of checking gastric residuals and caretaker hygiene (handwashing).

PROBIOTICS: THE CONTROVERSY

The definition of probiotics as defined by the World Health Organization is "live microorganisms which when administered in adequate amounts confer a health benefit on the host." Of interest, this definition implies a health claim and thus has triggered concern by safety authorities such as the European Food Safety Authority and the United States Food and Drug Administration (FDA).[44] This concern becomes even more acute when considering claims for prevention of diseases such as NEC in a highly vulnerable population such as preterm infants.

The history of "probiotics" predates use of this term. Fermented food products have been used for centuries worldwide, but the relationship of microbes to these fermented foods was poorly described until the beginning of the 20th century, when Eli Metchnikoff, the Russian scientist and Nobel laureate suggested that certain microbes may benefit the host by interfering with factors associated with the aging process. For the past several decades, the food industry has been a primary advocate for the widespread use of probiotics. Whether the paradigm of probiotics as a food to prevent NEC in preterm infants is appropriate needs to be questioned. This will be addressed in greater detail later in this review.

One of the first probiotic studies in neonates evaluated the effects of adding Lactobacillus to formula on the growth of intestinal microorganisms including pathogens.[45] The results showed there were no differences in the colonization patterns between the Lactobacillus treated and the control infants. In the past two decades several additional studies evaluated the effects of probiotics on neonatal outcomes including NEC.[46–49] Some of the initial studies relied on historical controls, but suggested benefit. Subsequent relatively small trials of probiotics for the prevention of NEC were done in preterm infants. Meta-analyses of these studies that have shown positive effects, which stimulated interest in this area.

The most controversial which was published in 2010 [50] along with a commentary that suggested that it was no longer necessary to do further clinical trials on probiotics.[51] Questions were subsequently raised about the quality of the studies comprising the meta-analysis.[46,52–55] Further, and most important, is the fact that 10 different forms of probiotics were used in the individual studies rather than a single agent. This was subsequently followed by a Cochrane review which included 16 studies that yielded similar results. [56] Although not a direct analogy, this approach is not much different than performing a meta-analysis that draws from a series of studies that utilize 10 different antibiotics to treat pneumonia, and subsequently concludes that we should equally consider treating with ampicillin or chloramphenicol based on a few studies that demonstrated benefit. Of course, these are all antibiotics but their spectrum of activity and safety is different. This raises several questions: Which probiotic might be the most beneficial; what dosage should be used and when should they be started; should larger studies that actually have adequate sample size based on the *a priori* hypothesis of NEC prevention be done? If one does a sample size determination based on a 5% baseline NEC rate, a 30% decrease with the intervention, and corrects for possible dropout rate and uses strict criteria for NEC diagnosis, none of the studies reported in the meta-analyses had an adequate sample size (nearly 2,000 needed in each arm of the study). Again, one of the strongest and most valid concerns raised pertains to the number of different agents in the studies that were meta-analyzed and the validity of combining agents with potentially different properties and mechanisms. In addition, safety concerns should also have been raised. In one of the largest studies, the smallest babies (<750 grams) had higher sepsis in the probiotic group.[57]

IS THERE A NEED FOR REGULATION OF PROBIOTICS FOR USE IN NEONATES?

There remains heated debate on whether probiotics should be used routinely in preterm infants for the prevention of NEC. Although the prevalence is not known, many neonatologists have started prescribing probiotics in NICUs for the prevention of NEC. Anecdotally, some of the agents being used are not even probiotics previously studied in preterm neonates. In the United States, there appear to be no probiotics that are “licensed” or for which there are well developed access schemes detailing routine use in the prevention of NEC as suggested by one author for their use. [51] There are also no current standards for “quality control of a reconstituted product”. Good manufacturing practices (GMPs) specifically for use of probiotics as drugs to prevent a specific disease such as NEC are not available. Of note is the fact that regulatory agencies in the United States and Europe have strict criteria for studies and the use of certain agents for which specific health claims such as prevention of NEC, are made. These include well controlled randomized studies that are adequately powered and studies of both safety and efficacy.

At this juncture, such studies have not been done in North America for probiotics. One of the largest (but still underpowered) studies to date was done in in South America where 750 babies ≤ 2,000 grams were evaluated with the probiotic *Lactobacillus reuteri* versus controls.[49] This study resulted in no difference in mortality, nosocomial infection or NEC between the probiotic treated and control infants. Another large multicenter randomized

study performed in Australia used 3 different microbes in the probiotic preparation and employed late onset sepsis (not NEC) as the primary outcome.[48] Approximately 1099 babies were evaluated, and their results showed that there was no difference in sepsis or the mortality rate between the two groups. These results, as in the study with *L. reuteri*,[49] did not support a decrease in mortality as seen in the meta-analysis.[47] However on secondary analysis in the Australian study there was a decrease in the incidence of NEC in babies >1,000 grams birthweight, but not in the smaller infants. The number needed to treat was 43 with a 95% CI 23–333. [48] Thus the question arises whether this actually incurs any true benefit if all preterm infants are treated routinely for the prevention of NEC.

From the perspective of the regulatory agencies, probiotics have not been treated as a drug but rather as a food, thus not being scrutinized with the same standards of a drug. This raises the question of quality and consistency of the product. It also needs to be remembered that probiotics are not a classic drug in that they are live agents that can proliferate and stay in the individual's GI tract indefinitely, as seen in recent studies of the intestinal microbiome after C-section versus vaginal delivery.[58,59] Furthermore, although authors of the meta-analyses suggest probiotics in preterm are safe, some studies on pancreatitis prophylaxis in adults[60], studies in preterm piglets [61] as well as the results in the ELBW infants (increased sepsis, IVH+PVL)[57] provide evidence that we cannot ignore that support the need for caution. It is also clear that "probiotics safety should be considered on a strain by strain basis".[62] Stringent standards for detecting microbial infections using culture media specific for the probiotic bacteria or specific PCR techniques have not been done in these studies. As stated by several authors [53,54,63–66] as well as by the AAP[67] and ESPGHAN[68] committees on nutrition, despite showing promise, additional studies with adequate sample size and with an approved product as per FDA (or equivalent for other countries) are advised.

DO WE HAVE ALTERNATIVES TO PROBIOTICS?

As described in the previous sections, it is very possible that probiotics may play a role in the prevention of NEC in premature babies. However, if large adequately done randomized control trials do not demonstrate safety and efficacy with certain probiotic preparations, it is unlikely that this will be repeated for several other single preparations. Thus we need to begin to consider options for the use of probiotics in the prevention of NEC.

As previously mentioned, the use of the human milk is a known preventative measure. Of interest is the fact that recent studies have shown that there are microbes present in human milk, [42,43] and that these microbes may originate from the maternal gastrointestinal tract. In one study, microbes from each mother were stable over time but differed considerably when comparing one mother's milk to the other others milk microbes.[42] This may suggest a specificity of the microbial composition of human milk for each mother's baby. Thus we may already be providing "probiotics" for the infants in their own mothers' (but not pasteurized donor) milk.

Several studies done in vitro and with animals suggest that certain components of microbes may stimulate the innate mechanisms of the gastrointestinal immune system and lead to a response that is actually protective in the gastrointestinal tract. [69] Thus it is possible that heat

inactivated or ultraviolet radiation inactivated microbes may benefit the host in terms of the prevention of an overtly active inflammatory response.[70] Certain molecules derived from the bacteria such as polysaccharide A (PSA), primarily derived from the Bacteroidetes phylum, may drive the conversion of naïve CD4 T cells to the production of regulatory T cells which are active in the production of IL- 10 and transforming growth factor beta (TGFβ), which are important immunomodulatory molecules.[71]

The carbohydrate composition of milk that is provided to preterm babies may also be of importance in the regulation of microbial growth and NEC. Although not considered as a classic prebiotic, one agent that is present in human milk in large quantities is the disaccharide lactose. Even though it is thought that preterm infants may have difficulties in the hydrolysis of lactose because of immaturity and local activity of the brush border enzyme lactase,[72] it is known that microbial fermentation of lactose in the distal small intestine leads to the production of the short chain fatty acids such as acetate, propionate, and butyrate, the latter of which is critical in the energy metabolism of the colonic epithelial cells and is also important in proliferation, differentiation and maintenance of junctional epithelial integrity.[73,74] Thus, if a formula that is devoid of lactose is given to preterm infants; this benefit may not be incurred.

Classically, prebiotics are non-digestible food ingredients, usually complex carbohydrates that cannot be digested by the human host, but can be utilized to stimulate the growth of beneficial intestinal bacteria. Several studies have shown that fructose oligosaccharides and glucose oligosaccharides (prebiotics) may actually soften stools and decrease crying in infants.[75,76] However at this juncture there are no studies that show the prebiotics are effective in the prevention of NEC. Human milk has also been found to be a rich source of oligosaccharides.[77,78] Human milk oligosaccharides (HMOs) have been the focus of intensive investigation and studies in animal models suggests that HMOs may be beneficial in the prevention of intestinal injury.[7980]Research is ongoing that may lead to their application in preterm infants.

Another area of major interest relates to fecal microbial therapeutics, one variant of which is the fecal transplant.[28] This has received considerable attention over the past decade largely because it has been effective in the treatment of the refractory *Clostridium difficile* infections. [81] There is emerging interest in using fecal transplants (or some variant) in ulcerative and Crohn's colitis, Type I diabetes, and prevention of NEC in preterm infants. [82–84] However this is a very controversial area, and the logistics of this modality remains a challenge.

THE FUTURE

It is clear that routine use of probiotics for prevention of NEC remains a highly controversial topic. There are currently neonatologists who insist on using probiotics without additional safety and efficacy studies, nor do these neonatologists consider of choice of probiotic strain. The need to know which probiotic or strains to use is of the utmost importance because they are not all the same, just as not all antibiotics are the same. Meticulously designed studies that are adequately powered and controlled to test the safety and efficacy of

individual probiotics are either underway or being planned with close collaboration of the regulatory agencies such as the FDA. Once the studies are completed, it will be clearer that the product(s) being provided are truly safe and beneficial. In the meantime, basic research on the developing microbiome and its interaction with the host will add to our understanding of how it might be safely manipulated to prevent diseases such as NEC in the neonate.

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Key Points

- It is clear that routine use of probiotics for prevention of necrotizing enterocolitis (NEC) remains a highly controversial topic.
- There are currently neonatologists who insist on using probiotics without additional safety and efficacy studies.
- Basic research on the developing microbiome and its interaction with the host will add to understanding of how it might be safely manipulated to prevent diseases such as NEC in the neonate.