FOCUS

A diverse balanced microbiota is necessary for the development of an appropriate innate and adaptive immune response

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Initial Intestinal Colonization in the Human Infant and Immune Homeostasis

by W. Allan Walker

Key insights

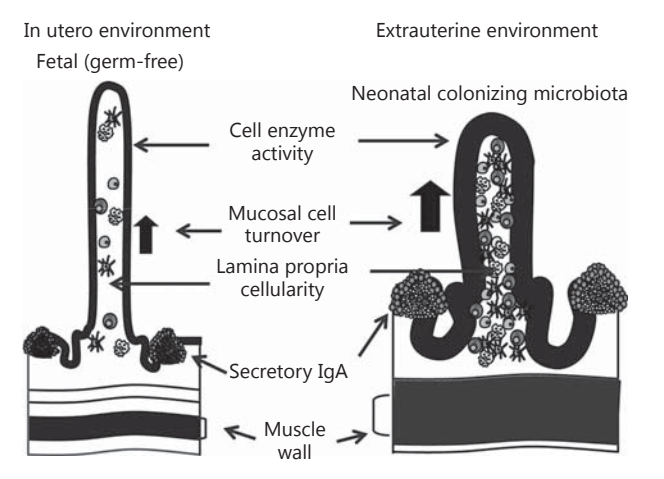
Initial colonization of the infant gut is an important event in the development of mucosal immune homeostasis. To this end, the infant's diet plays a major role in shaping the identity of the intestinal flora that will ultimately orchestrate innate and adaptive immune function.

Current knowledge

Bacterial colonization of the infant gut occurs in several distinct phases, starting with birth, followed by the introduction of oral feeding and weaning. Intestinal microorganisms establish a symbiotic relationship with the epithelial and lymphoid tissues of the host, evoking the development of the innate and adaptive immune response. Pattern recognition receptors, such as those of the toll-like receptor family, interact with bacterial molecules to trigger specific pro- or anti-inflammatory signaling cascades. The maturation of mucosal immunity is completed with the process of oral tolerance.

Practical implications

Normal colonization of the infant gut occurs when full-term infants are born by vaginal delivery and are exclusively breastfed for the first 6 months of life. Nutrition is a key environmental factor that influences the bacterial signature that will prevail throughout later life. The oligosaccharide content of human breast milk facilitates the growth of beneficial bacteria, providing the optimal environment to boost mucosal immunity. Factors such as caesarian delivery and excessive antibiotic usage can have a negative impact on early microbial colonization. The use of pre- and probiotics supports normal microbial flora, thereby restoring immune function in cases of aberrant microbial homeostasis as in necrotizing enterocolitis, infections and immune dysfunction.



The influence of bacterial colonization on intestinal function. A cross-section of the immature small intestine of a human fetus in utero (left) versus an identical section of the small intestine in the newborn infant in the extrauterine environment (right). One main difference between these two environments is that the intrauterine environment is germ free, whereas the extrauterine environment consists of abundant microbiota which colonize the gastrointestinal tract. Reprinted with permission from Walker [Funct Food Rev 2009;1:13–19].

Recommended reading

Weng M, Walker WA: The role of gut microbiota in programming the immune phenotype. J Dev Orig Health Dis 2013;4:203–214.

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Initial Intestinal Colonization in the Human Infant and Immune Homeostasis

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Key Messages

- **Initial bacterial colonization is in part determined by the infant's diet.**
- **A symbiotic bacteria-host relationship determines mucosal immune homeostasis.**
- **Abnormal colonization (dysbiosis) and its accompanying increase in disease expression can be prevented by pre- and probiotics.**

Key Words

Intestinal colonization · Immune homeostasis · Breastfeeding · Probiotics

Abstract

The paradigm of disease burden in the developed world has changed drastically in the last few decades from predominately infections to immune-mediated diseases (autoimmunity and allergy) because of alterations in the Western lifestyle (improved sanitation, immunizations, antibiotic usage and altered dietary intake). A diverse balanced microbiota is necessary for the development of an appropriate innate and adaptive immune response. There is strong evidence that disruption of the normal colonization process can lead to alterations in the important symbiotic relationship that is necessary for immune homeostasis. For example, infants born by cesarean section or receiving excessive perinatal antibiotics have inadequate initial colonization and aberrant mucosal immune function. As a result, later in childhood, they express an increased incidence in asthma and autoimmune diseases (e.g. celiac disease). An important component of initial colonization is the infant's diet. Breast milk contains a variety of nondigestible oligosaccharides which function as prebiotics preferentially stimulating proliferation of *Bifidobacteria* and *Lactobacilli*, important health-promoting bacteria, and cause fermentation of the oligosaccharides into short-chain fatty acids. In the absence of breastfeeding for the first 6 months of life, formula containing pre- and probiotics may overcome an initial inadequate colonization process and help establish a normal mucosal immune system.

Introduction

In this review, I will consider how the newborn, full-term, vaginally-delivered infant initially colonizes its gastrointestinal tract [1]. With full colonization, a symbiotic relationship develops between colonizing bacteria and the underlying epithelial and lymphoid tissues [2]. This relationship results in both nonspecific and immunologic (innate and adaptive immune responses) defenses which collectively comprise the intestinal mucosal barrier to pathogens and anoxious antigens [3]. An important component of mature intestinal immune homeostasis is the develop-

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ment of oral tolerance to benign commensal bacteria and anoxious antigens [4]. This phenomenon can be achieved with complete colonization of the gut during the newborn period [5]. With complete colonization and development of the mucosal barrier, immune homeostasis occurs and there is no expression of disease. In contrast, circumstances exist in which inadequate colonization occurs (premature delivery, delivery by cesarean section and excessive use of perinatal antibiotics) [6]. Under these conditions, an inadequate colonization occurs leading to dysbiosis and increased expression of immune-mediated and allergic disease states [7]. This dysbiosis of the gut has become the basis for the 'new' hygiene hypothesis. Fortunately, clinical evidence suggests that pre- and probiotics can act as 'surrogate' colonizers and help prevent the expression of these diseases [8]. Each of these concepts will be discussed in detail in this review.

Normal Initial Bacterial Colonization

A cross-section of the small intestine in the human fetus in utero appears as an immature epithelial surface with prolonged cell turnover and a paucity of lymphoid elements [1]. In contrast, an identical section of the small intestine in the newborn infant in the extrauterine environment appears as an active structure with a rapid turnover, expressing the subtypes of epithelial cells and displaying a plethora of lymphoid elements (fig. 1) [2]. The principal difference in these two situations is that the intrauterine environment is germ free, whereas the extrauterine environment consists of abundant microbiota which colonize the gastrointestinal tract. This observation emphasizes the importance of initial intestinal colonization in the development of gastrointestinal functions.

Thus, normal initial colonization of the gut is an important event in the adjustment of the newborn to the extrauterine environment [9]. Several factors influence initial intestinal colonization. These include the infant's genetic signature, the nature of the delivery process, the use of excessive antibiotics during the perinatal period and whether the mother is under stress or expresses an inflammatory condition [10]. Normal colonization is most likely to occur when the infant is born full term by a vaginal delivery and is exclusively breastfed during the first 6 months of life (table 1). Colonization occurs in phases over 1 year to 18 months in the postpartum period. The full-term infant leaves the germ-free intrauterine environment and passes through the birth canal where it ingests a healthy bolus of maternal vaginal and colonic microbiota. This represents the first and most important

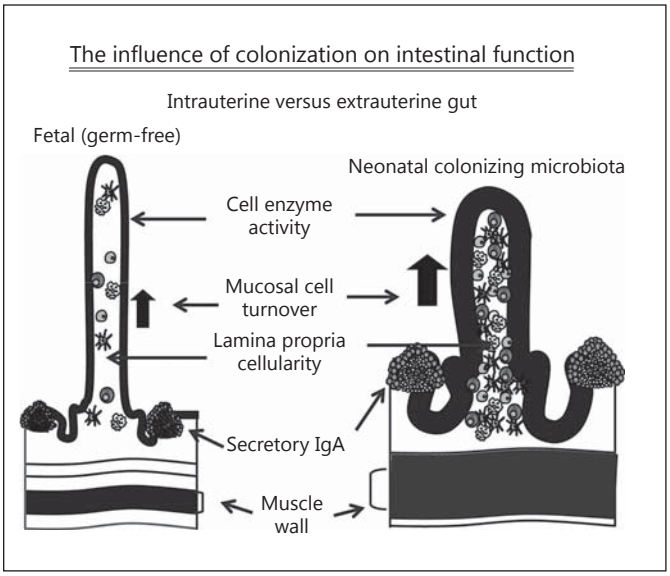

Fig. 1. A schematic representation of a cross-section of the small intestine of a human fetus in utero versus a newborn human infant. The fetal intestine appears thin and exhibits a slow epithelial proliferation rate with a paucity of gut-associated lymphoid tissue (GALT), whereas the infant intestine manifests a robust, diverse epithelium with a fast turnover rate and abundant GALT elements. Reprinted with permission from Walker [1].

Table 1. The phases of initial bacterial colonization

Normal colonization	
–	'Germ-free' intrauterine environment
– Phase 1:	acquire maternal vaginal/colonic flora (full-term vaginal delivery)
– Phase 2:	introduction of oral feedings (breast milk vs. formula)
– Phase 3:	weaning
– Phase 4:	acquire complete adult colonization (12–18 months; more than 1,000 species)

phase of colonization [11]. With the introduction of oral feedings, the ingested bolus is further stimulated (phase 2). The nature of initial oral feeding, e.g. breast versus formula feeding, has profound short-term effects on the composition of colonizing bacteria [12]. The role of early nutrition in bacterial colonization will be discussed in greater detail later in this review. At the time of weaning to complementary foods, e.g. after 6 months, colonization is further effected (phase 3). Finally, by 1 year to 18 months of age, the infant's intestine is completely colonized with a unique signature of microorganisms consist-

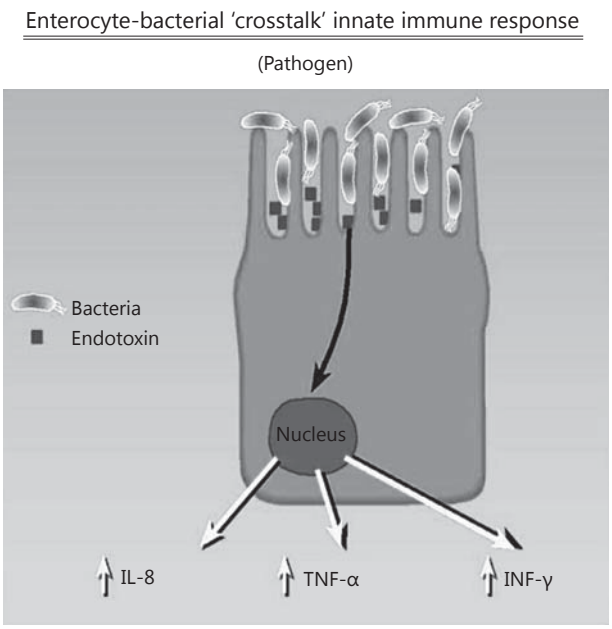

Fig. 2. Pathogens in the intestinal lumen bind to PRRs (TLR2 and 4) on enterocytes to evoke an innate immune response via signaling molecules and transcription factors leading to a self-limited inflammatory (IL-8, TNFα, etc.) response to prevent bacterial penetration.

ing of more than 1,000 separate species and more cells by 10-fold than cells in the human body [13]. If antibiotic treatment is used during this period, the timing and nature of colonization is disrupted and prolonged [14].

A fully colonized intestine can function as an ancillary organ in the body. It consists of 1–2 kg of body weight in the human adult and has a 10-fold greater number of cells than the cells of the human body as well as a 100-fold greater number of genes than the human genome. Furthermore, the metabolic activity of colonizing bacteria is greater than the most active body organ, namely the liver [15]. Accordingly, investigators over the last decade have expanded exponentially our understanding of the functions that colonizing microbiota have in human body function, particularly intestinal and immune function [16].

Symbiosis and Immune Function

Once a normal colonization has been achieved with diverse individual bacterial species, these microorganisms establish a symbiotic relationship with the intestinal epithelial and lymphoid tissues. Conserved molecular patterns, either expressed on the surface of symbiotic bacteria or secreted into the gut, can interact with pattern recognition receptors (PRRs) expressed on or inside epithelial and lymphoid cells to initiate signal transduction and transcription of a host of molecules which mediate host defense or metabolic activities within the intestine [17]. The best-known family of PRRs is the toll-like receptor (TLR) family consisting of 9 identified receptors which interact with components of Gram-positive and Gram-negative bacteria to mediate both innate and adaptive immunities as well as other mucosal barrier cellular functions [18].

Innate Immune Function

Colonizing commensal and pathologic organisms can interact with TLRs on the intestinal epithelial cell to evoke an innate immune response. For example, lipopolysaccharides on the surface of Gram-negative organisms, particularly pathogens, stimulate TLR4 by binding to a lipopolysaccharide-binding protein and a surface molecule CD14 on the enterocyte surface [19]. An ancillary protein (MD_2) helps to anchor the complex to TLR4 which then activates signaling molecules that allow for the transcription factor NFκB to disassociate from its binding protein IκB in the cytoplasm and traverse the nucleus to activate inflammation, through the transcription of cytokines and chemokines that in turn mediate inflammation. Inflammation prevents bacterial penetration across the epithelium and into the blood stream leading to sepsis [19]. This inflammatory innate reaction to pathogenic bacteria is spontaneous and self-limited in order to prevent chronic inflammation (fig. 2). With sustained interaction between the molecular pattern and its PRR, negative regulators of inflammation are activated to inhibit inflammatory signaling at various steps along the innate immune pathway [20].

Adaptive Immunity

In like manner, colonizing bacteria can activate adaptive immunity to create immune homeostasis within the intestine. For purposes of illustrating this phenomenon, three examples of adaptive immunity will be considered (table 2). Polymeric IgA (pIgA) produced by B cells within mesenteric lymph nodes is secreted onto the intestinal surface and acts as 'aseptic paste' to protect against invasion by pathologic organisms or noxious antigens (table 3). At birth, full-term, vaginally-born infants are pIgA deficient [21]. It takes a finite period postpartum (1 month) for protective levels of pIgA to appear. The matu-

ration of pIgA corresponds to the first and second phases of colonization (table 1). A classic publication [22] has shown the mechanism of this process. Colonizing bacteria within the lumen are taken up by dendritic cells that penetrate through appendages between enterocytes into the lumen or underlying microfold cells over Peyer's patches. Engulfing dendritic cells then migrate to the mesenteric lymph node where they present the engulfed bacteria to B cells to activate them into pIgA-producing plasma cells. Secreted pIgA directed against engulfed microbiota are in turn transported to the intestinal surface where they coat the microvillus membrane to protect against invasion [21].

An important component of immune homeostasis is to have a balanced T helper (Th) cell response. Colonizing microbiota help to ensure that this happens. This occurs through 'crosstalk' between luminal bacteria and the TLRs on dendritic appendages penetrating into the intestinal lumen. Activation of dendritic cells results in their producing a cytokine environment which allows naive Th cells (Th0) to mature into Th1, Th2, Th17 and T-regulatory (Treg) cells (fig. 3) [23]. Th1 cells mediate cellular immunity, and Th2 cells mediate humoral immunity (e.g. antibody production) including the production of IgE antibodies. A new subclass of Th cells, Th17, mediates tissue inflammation and clearance of extracellular pathogens. Probably the most studied Th subclass over the last few years are Treg cells (TR1 and Th3) which mediate oral tolerance and anti-inflammation [24].

Oral Tolerance

Maturation of mucosal immune function leading to immune homeostasis is not complete until the process of oral tolerance occurs. Oral tolerance is a systemic reduction in cellular and humoral immunity to commensal bacteria and anoxious antigens through exposure to the intestine via the perioral route. Figure 4 depicts our current understanding of oral tolerance. Antigens or nonpathogenic bacteria interacting with submucosal dendritic cells via TLRs in the presence of colonizing bacteria are stimulated to preferentially produce Treg cells and a specialized microenvironment that facilitates the development of Treg cells. These cells release TGF-β, an oral tolerogenic cytokine, which reduces the Th1, Th2 and Th17 response to antigens/bacteria [25]. It has previously been shown that oral tolerance cannot be achieved in germ-free animals [26] and these animals must be conventionalized to full colonization during the neonatal period for tolerance to be effective [27]. In our laboratory, we have shown that oral tolerance requires an intact TLR4 to be effective, and tolerance can be broken with extensive use of broad-spectrum antibiotics [26]. These observations suggest that normal initial intestinal colonization is needed to establish oral tolerance, and tolerance once achieved can be broken by excessive use of antibiotics.

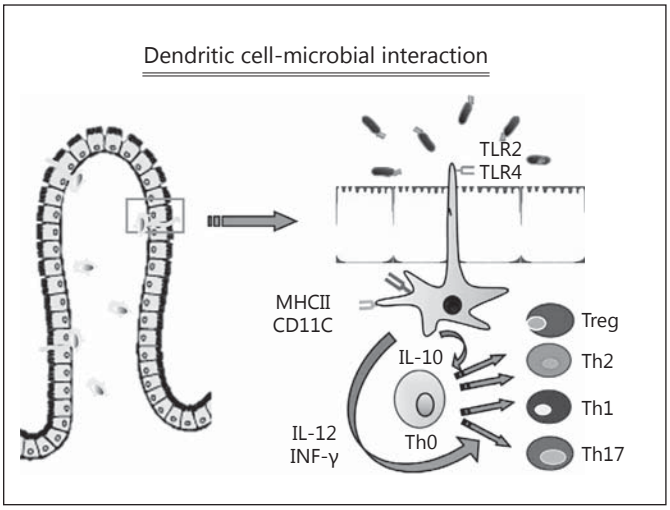

Fig. 3. Commensal bacteria evoke T cell responses via dendritic cells by binding to TLR2 and/or TLR4 on the surface of penetrating dendrites. Commensal microbiota induce MHCII$^+$ CD11C$^+$ dendritic cells to secrete IL-10 or IL-12 and INF-γ by which naive Th0 cells are primed to differentiate into Th1, Th17 or Treg, respectively. Reprinted with permission from Walker [1].

Table 2. Adaptive immunity

– pIgA secretion
– Balanced Th cell response
– Oral tolerance

Table 3. Microbiota and mucosal host defense

Initial abnormal bacterial colonization		
–	Phase 1:	sparse, inadequate colonization premature delivery cesarean section use of prophylactic antibodies
–	Phase 2 and 3:	introduction of feeding results in slight modification
–	Phase 4*:	delayed incomplete colonization until 4–6 years

*More susceptible to pathogens and immune-mediated disease, e.g. allergy.

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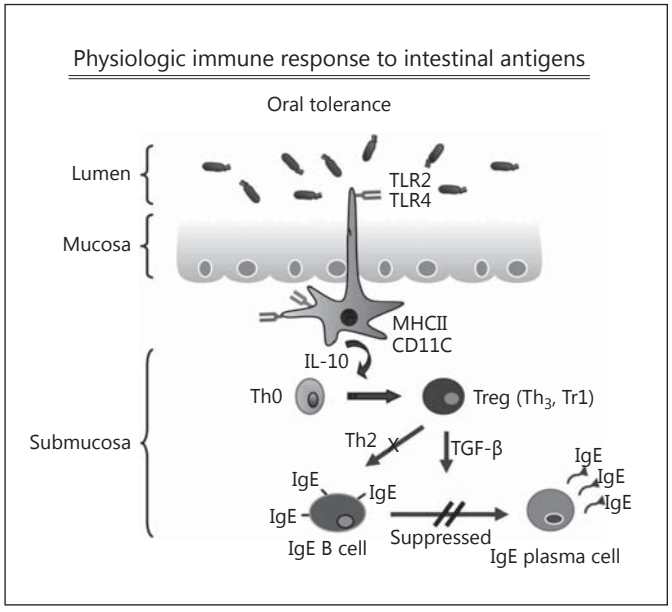

Fig. 4. Schematic representation of oral tolerance induction by gut microbiota. In the intestinal lumen, gut microbiota activate dendritic cells via the TLR2/TLR4 signaling pathways. Activated dendritic cells cause maturation of Th0 to subsets (Th3, Tr1) of Treg cells via release of IL-10 to stimulate TGF-β release and thereby suppress IgE production. Reprinted with permission from Weng and Walker [2].

Infant Nutrition and Initial Colonization

As stated in the introduction to this review, nutrition is an important environmental factor that influences the composition of colonizing bacteria. At no other time in life is nutrition as influential in determining colonization as it is during the newborn period when the infant is initially establishing its lifelong signature of microbiota. Striking short-term differences in colonizing bacteria occur if the newborn infant is exclusively breastfed compared to formula feeding [28]. Breastfed infants during the first month of life have an increase in health-promoting bacteria *(Bifidobacterium infantis, Lactobacillus acidophilus* and *Bacteroides fragilis)* [29]. This was first shown using conventional culture techniques almost 30 years ago [28]. More recently, using metagenomic analysis of infant intestinal contents, it has been shown that breastfed versus formula-fed infants have differences in large families of bacteria (phyla) and more diversity in individual species [29]. Moreover, bacteria stimulated by breastfeeding activate more biologically and immunoprotective genes in the host than by formula feeding [30]. Although not as striking in its effect, diet after weaning and in early childhood over long periods of time can continue to affect bacterial phyla and individual species [31]. It is now suggested that the dietary influence (Western diet) on bacterial colonization may be an important factor in the paradigm shift in disease burden in developed countries from predominately infection to immune-mediated (autoimmune and allergy) diseases [32].

A principal component of influence on colonizing bacteria relates to the oligosaccharide content of breast milk [33]. Oligosaccharides make up 8% of the total nutrient content of human milk. They are not digested in the small intestine but enter the colon where they are fermented by colonic bacteria leading to an acid milieu and an increase in short-chain fatty acids (prebiotic effect). This results in a boost in health-promoting bacteria (e.g. probiotic bacteria) and an early stimulus to mucosal immune defense. In fact, a previous clinical study has shown a direct association with the pIgA levels in the intestine during the first months of life and the number of *B. infantis* organisms present [34] and an inverse relationship between levels of *B. fragilis* and the inflammatory cytokine IL-6, suggesting an anti-inflammatory effect [34]. In fact, recent studies measuring the stimulation of *B. infantis* genes (*B. infantis* has had its genome sequenced) when grown on human milk oligosaccharides (HMO) versus artificial prebiotics (inulin, fructooligosaccharide or galactooligosaccharide) have shown a striking difference in gene response [35]. Subsequent studies have suggested that HMO-grown *B. infantis* genes can actively stimulate increased expression of tight-junction proteins and also provide anti-inflammatory effects [36]. These studies collectively suggest that breast milk nutrition in infancy is critical to early colonization of the newborn gut and to the development of mucosal immune-protective function.

At no other time in life is nutrition as influential in determining colonization as it is during the newborn period when the infant is initially establishing its lifelong signature of microbiota.

Abnormal Initial Bacterial Colonization

Table 3 depicts the conditions in which abnormal initial bacterial colonization occurs. Disruption of phase 1 can occur in premature delivery, delivery by cesarean section and with excessive use of perinatal antibiotics. In each of these circumstances, there is an inadequate initial

Table 4. Clinical consequences

– NEC – with prematurity
– Asthma – with antibiotics
– Inflammatory bowel disease – with antibiotics
– Atopic disease – with cesarean section

colonization. Despite the stimulus of oral feeding and weaning, final colonization can be delayed until 4–6 years of age during which time the infant is more susceptible to both infections and immune-mediated disease states. Inadequate colonization leads to a dysbiosis of intestinal microbiota and the intestine which in turn leads to immune dysfunction and an increased tendency for inflammatory disease [2]. In fact, many chronic intestinal conditions, e.g. necrotizing enterocolitis (NEC), allergy and inflammatory bowel disease, have been shown to be associated with an intestinal microbiota different from age-matched non-disease controls [37]. Furthermore, when germ-free mice are colonized with the microbiota from patients with allergic, obese or malnourished conditions, they develop a phenotypic expression of the actual disease [38], suggesting that a dysbiotic microbiota may contribute to symptoms of disease.

Diseases Associated with Intestinal Dysbiosis

Several clinical conditions have been associated with the increased expression of disrupted colonizing bacteria (table 4). For example, NEC represents a condition in premature infants in which the colonizing bacterial phyla are not equally represented compared to age-matched controls and diversity of individual species is lacking [39]. We have also shown that the fetal intestine, like the mature intestine, responds to both pathogens and commensal bacteria with an excessive inflammatory response and that this is due to a developmental expression of innate inflammatory immune response genes which favor an inflammatory response to all colonizing bacteria [40]. We have moreover reported that a fetal cell line, fetal organ cultures and intestinal fetal xenografts respond excessively to exogenous and endogenous inflammatory stimuli [41] due to an overexpression of TLRs, signaling molecules and transcription factors and an underexpression of negative regulators [42]. In like manner, increased episodes of antibiotic treatment, particularly a broad-spectrum antibiotic treatment in the first year of life, have been associated with an increased expression of asthma [43] during adolescence and inflammatory bowel disease [44] during childhood. Furthermore, women with a history of atopic disease deliver infants that are 8-fold more likely to express allergy if they are born by cesarean section rather than by vaginal delivery [45]. These clinical studies strongly suggest that an abnormal intestinal bacterial colonization leading to dysbiosis can increase the incidence of immune-mediated disease.

Prebiotics and probiotics or a combination, e.g. symbiotics, may convert a dysbiosis to a symbiosis by balancing potential pathogens with health-promoting bacteria.

Pre- and Probiotics Are 'Surrogate' Colonizers

Fortunately, there are possibilities of dealing with dysbiosis leading to clinical disease. Several clinical studies have been published which suggest that prebiotics and probiotics or a combination, e.g. symbiotics, may convert a dysbiosis to a symbiosis by balancing potential pathogens with health-promoting bacteria. Two circumstances illustrate this approach to rectifying a dysbiosis of intestinal microbiota. A seminal study from Finland [46] has shown that when *Lactobacillus rhamnosus* (LGG) is given to pregnant women with a family history of allergy during the latter stages of pregnancy, this results in infants with a 50% lower incidence of atopic dermatitis than control infants. Furthermore, this protective effect is still apparent at 7 years after birth [47]. However, when these studies were expanded to include multiple test sites using a single protocol, the results were not as clear-cut [48]. Yet, the probiotic used during pregnancy and lactation was helpful if the allergy-prone babies were born by cesarean section. Another example of probiotics stabilizing a dysbiosis occurs with their use in premature infants to prevent NEC [49]. Several studies have been done and when analyzed by a meta-analysis seemed to both prevent and lessen the severity of NEC [50]. A study performed in Taiwan initially used a combination of *L. acidophilus* and *B. infantis* in one nursery to significantly reduce the incidence and severity of the disease. This was followed by an expanded study in five nurseries with similar results [51]. Since the Food and Drug Administration (FDA) in the United States will not allow live organisms to be given to immune-compromised premature infants, we have tested in human fetal intestinal models the effect of secreted

products of these two bacteria and then secretions from each grown separately. We have reported that secreted products of *B. infantis* have greater anti-inflammatory properties than those of *L. acidophilus*, and the anti-inflammatory function seems to be mediated through the stimulation of immature genes in the innate inflammatory immune response [52]. Further studies are planned to test the secreted factor with expressed breast milk from mothers delivering premature infants to determine if this combination of pre- (breast milk) and probiotic secretions may be protective.

Other examples of a prebiotic protective effect on dysbiosis suggest that when given after birth, prebiotics and probiotics appear to be protective against mild infections occurring during the first 6 months and allergy symptoms in allergy-prone infants during the first 2 years [53], as well as causing an enhancement of specific antibody levels with vaccines for polio [54] and *Salmonella* [55].

Summary and Conclusions

In this review, it was emphasized that initial colonization was in part dependent on the infant diet, particularly breastfeeding. Furthermore, it was shown that a symbiotic bacterial-host relationship determines immune homeostasis. An important component of immune homeostasis is the development of oral tolerance which can only occur with complete colonization of the intestine. Under conditions of abnormal colonization (dysbiosis), an increase in immune-mediated disease occurs. Fortunately, pre- and probiotics given to the infant can convert a dysbiosis to a symbiosis and potentially reduce the incidence of disease.

Disclosure Statement

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