Intestinal Immune Health

Michelle E. Conroy, W. Allan Walker

Mucosal Immunology and Developmental Gastroenterology Laboratories, Massachusetts General Hospital for Children, Department of Pediatrics, Harvard Medical School, Boston, MA, USA

Abstract
The fetal intestinal immune system is structurally intact from a very early gestational age. At birth, the neonate is challenged with an extraordinary and variable bacterial challenge. This mucosal and bacterial interface is the site of critical symbiotic and potentially pathogenic interactions. Neonatal inflammatory reactions are often exaggerated, creating a situation in a newly colonized gut whereby homeostasis must be actively achieved. Fortunately, the neonate is armed with a multitude of protective mechanisms by which to ensure a productive microbiota in the setting of an intact mucosal surface. The intestinal epithelium orchestrates complex interactions and signaling through a variety of intrinsic and extrinsic stimuli. Chief among these is the immunomodulatory capacity of breast milk which is increasingly implicated in the achievement of intestinal and immunologic health via a multitude of mechanisms. Additionally, developmental expression of enzymes, pattern recognition, downstream signaling and dendritic cell interaction all contribute to intestinal homeostasis. Current research is uncovering the molecular mechanisms behind many of these mechanisms. These strategies lend insight into the establishment of tolerance so critical to neonatal health. In a clinic context of increasing food allergy and inflammatory bowel disease, elucidating this machinery is increasingly pertinent. Future research should explore these molecular interactions more closely for their potential therapeutic applications.

Introduction
Fetal development and the transition from the womb imply an elegant anatomic and physiologic preparation for drastic changes in environment and exposure. The immune system of the neonate requires both instant readiness in the event of perinatal infection but also education about its new surroundings. As a result, the infant is in the unique immune circumstance of readied ignorance. The intestinal mucosa can be likened to the neonate itself. Initially
sterile and then rapidly exposed to a completely novel environment, this single epithelial layer must quickly and effectively learn and react. But the larger challenge in this mucosal context lies in the challenge of how to reap the benefits of the vast luminal bacterial world while minimizing harm to the mucosa and the host at large. Therefore, the infant’s immune system must quickly establish a fine balance between Th1 and Th2 responses. Excess of either is well known to lead to either inflammatory diseases such as inflammatory bowel disease or atopic diseases, respectively. The kinetics of the immune system are being elucidated with inspiring rapidity. This review will incorporate both well-established and recent data to present an abbreviated depiction of fetal and neonatal mucosal immune development and some of the potential molecular mechanics driving gut homeostasis.

**Fetal Immune Structure and Function in the Gut**

Throughout gestation, the fetus undergoes predictably timed assembly of and protection by various immune system components and surrogates. In fact, the basic template of the mucosal immune system is established very early. Specifically, the intestinal villi are first observed at about 10 weeks and the crypts/stem cells at 10–11 weeks. Groupings of lymphocytes resembling Peyer’s patches are observable by about 100 days of gestation. These areas further develop into increasingly organized areas of B cell follicles and T cell zones by 130–140 days [1]. B cells within these lymphoid collections are sIgM+, sIgD+, and CD5+ with only occasional IgA+ and IgG+ cells. This exception continues through birth when the lamina propria remains devoid of these same immunoglobulin-specific cells. There are MHC-II-positive cells in this space, but the definitive cell type has not been defined [2]. It is also important to note the absence of germinal centers and, hence, B-cell proliferation throughout fetal life. It is also noteworthy that the intestine’s ability to produce IgA in either fetal life or early infancy is virtually nonexistent. As such, the basic components for mucosal immune function are present quite early in gestation. This is essentially the same for the systemic immune system, the development of which occurs in parallel with the mucosal system [for review see, 3].

Though maternal immunoglobulin dominates fetal and neonatal immunity, the fetus is capable of generating IgM and IgG. This occurs in the spleen and peaks at approximately 17 weeks of gestation. Despite this, immunoglobulin levels are low at birth and are virtually all maternal in origin [3]. Passive transfer of maternal antibody begins at approximately week 16 of gestation. The initial ratio of fetal to maternal immunoglobulin is quite low, but a subsequent increased concentration of immunoglobulins results in near maternal levels by the third trimester. Therefore the majority of transfer occurs late in gestation. Immunoglobulin receptors have been demonstrated on
placental tissue through radiolabeling. Studies of mRNA transcripts have demonstrated variable expression of the three different FcγR reflecting consistent expression of FcγRI and FcγRIII, but variable expression of FcγRIIb, which increases significantly in the second and third trimesters [4]. Notably, the FcγRIIb is typically not expressed on any adult endothelial surfaces other than the placenta. Recently, it has been shown that FcγRIIb associates with a novel IgG-containing intracellular organelle within placental epithelial cells. This may likely be a mechanism by which the fetus specifically accumulates maternal IgG, particularly given the temporal correlation between increased fetal immunoglobulin levels and expression of this particular receptor [5]. The fetus is thus protected passively via this transfer of maternal immunoglobulin.

While the fetal immune system has traditionally been regarded as quiescent, it is clear that it can mount adaptive, inflammatory immune responses. As an example, Hermann et al. [6] demonstrated impressive, oligoclonal T-cell expansion in the cord blood of newborns who had been congenitally infected with *Trypanosoma cruzi*. This expansion was accompanied by the expression of effector molecules including IFNγ and TNFα indicating competence of these T cells. A similar capability has been noted in response to other infectious agents, which has raised questions regarding the resting state of the fetal immune system. It has previously been demonstrated in vitro that immature human enterocytes demonstrate exaggerated IL-8 secretion via IL-1β and TNFα stimulation [7]. Therefore, mediators must exist that dampen this fetal tendency toward overt inflammatory responses. A regulatory safety net is implicated to protect the fetus and neonate from this immune reactivity. CD4+CD25+ T cells have been detected in fetal tissue at 13 weeks gestational age which occurs in tandem with other T-cell migration from the thymus. These regulatory cells were stable in number throughout gestation and birth. This is in contrast to other T cells which demonstrate variable levels and maturation evidenced through analysis of surface markers [8]. A recent study demonstrated a large population of CD4+CD25+ T cells present in cord blood and fetal mesenteric lymph nodes at higher levels than in adults. Subsequent removal of these cells from fetal mediastinal lymph node culture resulted in significant T-cell proliferation and IFNγ production. This cellular expansion and IFN production did not occur in adult tissue culture upon the removal of regulatory cells [9]. This striking difference between fetal and adult tissue highlights the critical importance of regulatory activity in establishing peripheral tolerance in the fetus.

Thus, as the neonate readies for birth and entrance into the contaminated world, the issue of potentially excessive inflammatory responses becomes critical. Clearly the fetal to neonatal transition must include means through which this inflammatory default must be mitigated. Much work has been done to understand the mechanisms behind this process which leads ultimately to the enigma of oral tolerance.
The Variable Inoculation of Birth

The amniotic environment is a sterile one that protects the developing fetus from infection. As a result, the fetus is presumably ‘sterile’ prior to birth. However, birth itself results in a massive introduction of bacteria, regardless of the mode of delivery. Significant differences exist in the microbial exposure and resultant colonization in babies born by cesarean section (CS) versus vaginal delivery (VD). Infants born vaginally acquire maternal resident vaginal and colonic microbes. The sterile conditions of surgical birth necessarily predict that environment will make a larger contribution to the babies born by CS. When fecal microbial content was evaluated from 3 days of age to 6 months of age, there were marked differences in VD versus CS infants. Most notably, there was no colonization with *Bacteroides fragilis* in the CS group before 2 months of age. By 6 months, the bacteroides colonization rate was half that of infants born by VD. Additionally, VD infants had greater colonization with lactobacillus and bifidobacter, whereas the CS infants were more colonized with clostridium. All of these results were significant [10]. This variance in mode of delivery and resultant colonization has clinical implications. CS has been cited as a risk factor for allergic disease. Specifically, CS has been associated with an increased risk of allergic rhinoconjunctivitis and asthma [11]. It has also been implicated as a risk factor for infantile diarrhea and IgE responses to food antigen including egg [12]. Therefore, even these initial bacterial interactions with the neonatal epithelium have lasting impact and effects. This bacterial proximity reinforces the intensive need for protection from the neonate’s tendency toward inflammatory reactions. Almost right away, there is another microbial onslaught via feeding. The ability to tolerate intake of novel dietary antigen is one of the major immunologic tasks of the neonate. While the mechanisms are far from understood, it is clear that the neonate is assisted via the interaction of the gut epithelium, commensal bacteria, and human milk. Inappropriate function at any of these interfaces may lead to inflammation or allergy. It is a great, delicate task of balance and resultant homeostasis.

The Feeding Frenzy

It is intuitive that feeding influences the microbial flora and antigenic stimulation of the developing intestine. The aforementioned initial colonization via birth is rapidly altered by the introduction of feeding. One study of 40 infants from days 3–21 of life demonstrated marked variability in colonization between breast- and bottle-fed infants. In this study, which confirms others, bifidobacter becomes the dominant bacteria by 1 week of age in breastfed infants. Bottle-fed infants show a much more diverse flora with a predominance of bacteroides [13]. Bottle-fed infants continue to show more diverse
and potentially pathogenic flora including clostridia throughout the first months of life. Thus, breastfed babies support more ‘beneficial’ microbial colonization. The mechanisms by which this occurs elucidates the protective nature of human milk. In a broad sense, human milk serves to ‘quiet’ the hyperactive inflammatory response of the neonate. There are multiple milk components that appear responsible for this modulation of neonatal inflammation. Human milk concentrations of TGFβ were shown to decrease TNFα-induced IL-8 secretion in vitro. This reduction was shown to be more dramatic in fetal intestinal epithelial cells than in more mature cells [14]. Soluble Toll-like receptor-2 (TLR2) is present in human milk as well as plasma [15]. It appears to be another mechanism through which excess inflammation may be avoided (fig. 1). Human milk also contains soluble CD14 (sCD14), a co-receptor for TLR4 recognition of LPS. Intestinal epithelial cell responses via TLR4 are dependent on the presence of sCD14. But other currently unknown milk components are responsible for enhancing cellular responsiveness to stimulation via TLR4. This milk factor likely assists the neonatal gut in its response to gram-negative organisms [16]. Human milk contains carbohydrates that are unique and reach the distal gut of the neonate structurally intact. This ultimately encourages synergistic microbial colonization which results in inhibition of harmful inhabitants [17]. It is therefore clear that the epithelial layer of the gut interacts with microbes and milk (human or artificial) products to establish protection and immune modulation for the neonate. These interactions, when appropriate, begin to create a process in which tolerance can be established. While the exact mechanisms of this are unknown, multiple epithelially based processes are being discovered that provide clues.

Fig. 1. sTLR2 inhibits cell activation. IL-8 and TNF-α production by Mono Mac-6 cells ($5 \times 10^4$) cultured in the presence of sTLR2. From LeBouder et al. [16].
As the infant is bombarded with billions of bacteria that are of variable potential pathogenicity, the epithelium must provide effective barrier protection. It has help from other mucosal cells including the antimicrobial proteins of Paneth cells and the mucous of goblet cells. There are also tight junctions between the epithelial cells, but these are not impenetrable as dendritic cells traverse through as do pathogenic bacteria. It turns out that the TLRs expressed on intestinal epithelia contribute to protecting the intact barrier. In MyD88−/− animals morbidity and mortality after administration of an epithelial toxic substance were marked and significant. The animals exhibited colonic bleeding and anemia which likely led to their increased mortality. Intact TLRs prevented this extensive epithelial damage, likely by the induction of pathways yielding protective and reparative factors such as IL-6 and heat-shock proteins [18]. The pattern recognition of colonized bacteria, then, likely assists the epithelium in maintaining a constitutive barrier to invasion. With one cell layer constituting such a critical separation, reparation via commensal stimulation is an efficient example of coexistence.

Another study also implicates TLRs and NFkB in the maintenance of epithelial integrity. NEMO knockout mice were shown to exhibit chronic intestinal inflammation. These mice also had decreased production of antimicrobial peptides and increased TNF-induced epithelial apoptosis. As a result, not only is chronic inflammation induced, but epithelial integrity is compromised leading to bacterial translocation and an additional stimulus for inflammation. Notably, mice bred to lack both NEMO and MyD88 did not exhibit this pathology. This clearly implicates the central role of epithelial TLRs in signal transduction leading to the eventual inflammatory state in the absence of these modifiers [19]. The neonatal gut barrier, then, is reliant on epithelial–bacterial interaction to maintain a strong barrier against invasive organisms.

From the moment of impact, initial bacterial docking, the epithelium has devised ways of regulating the colonization of the gut. Bacteria utilize cell surface glycoconjugates as receptors for epithelial adherence. In rodents, this is apparently under both regional and developmental regulation resulting in variability of terminal epithelial glycosylation by age and anatomical location [20]. This specifically relates to activity of sialyl- and fucosyltransferases which have predictable activity based on age and weaning. Most notably, germ-free animals do not appear to express these enzymes variably, regardless of age or weaning [21]. With the introduction of colonizing organisms, however, the germ-free animals express increased fucosyltransferase similar to their conventional counterparts (fig. 2). This relationship between bacterial
presence and epithelium function points again to the critical importance of proper initial and maintained colonization. A step further in logic suggests that alternative bacterial presence will result in a varied epithelial surface response. In turn, this may encourage less symbiotic and more pathogenic bacterial effect in the gut. This bacterial and epithelial interaction is compelling. Because of its circular nature, it again reinforces the pertinence of proper initial colonization.

**The Dynamic Duo**

The polarity of the epithelial layer creates the consummate separation of self and non-self. Various recent studies are elucidating the elegance of this proximity in bridging that discrepancy and allowing non-inflammatory coexistence. It seems increasingly clear that dendritic cells and epithelial cells are partners in the game of gut homeostasis. Rimoldi et al. [22] demonstrated that human gut dendritic cells show a bias toward the Th2 response, which seems logical given the proximity of microbes and the need to avoid chronic inflammation. But their studies implicated the epithelium in the generation of this tendency. Epithelial cells release thymic stromal lymphopoietin (TSLP) which influences dendritic cell maturation and leads to T-cell encouragement in the Th2 direction. TSLP is a product of NFκB transcription, the downstream effect of TLR stimulation by bacteria [22]. However, and perhaps more compelling within the same study, is that invasive bacteria reaching the basolateral side of the epithelial layer can induce TSLP production. When this
occurs, local TSLP levels are greatly increased as compared to levels in the noninvasive scenario. In this TSLP flood, dendritic cells regain the ability to release IL-12 and a Th1 response is elicited. The study included evidence that epithelial cells constitutively express TSLP but only when co-cultured with bacteria. This study gives evidence of another mechanism through which neonatal excessive inflammation is reigned via interactions between commensal bacteria, epithelium and downstream immune cells.

Other TLR intermediaries are also implicated in the task of inflammation modulation. Zaph et al. [23] showed that removal of epithelial Ikk, part of the TLR→NFκB pathway, resulted in an intensive inflammatory response in animal models. Ikk would normally activate NFκB with resultant downstream gene expression including TSLP which, as mentioned previously, yields a Th2 response. In the setting of a parasitic infection in Ikk-deficient animals, there was extensive damage via Th1 and Th17 responses, but no evidence of a protective Th2 response [23]. Interestingly, it is also the case that IκB is developmentally regulated. In immature intestinal epithelial cells, IκB levels were notably lower in immature cells. This was coincident with increased levels of IL-8 expression via bacterial component stimulation [24]. Taken together, these results point to another mechanism by which neonates tend toward inflammation. They also lift up the critical balance between and among immune cells at the gut interface (fig. 3).

But dendritic cells are hardly passive bystanders. As mentioned above, they can push through the epithelial barrier to sample bacterial antigen and induce the maturation of T cells via antigen presentation and cytokine milieu. However, like the epithelial cells, these cells extract advantage out of another kind of restriction. Specifically, dendritic cells take bacteria from the lumen only so far as the mesenteric lymph nodes. Here B- and T-cell stimulation and maturation can occur with subsequent re-homing to the gut. The systemic immune system is taken out of the commensal equation, thus compromising another example of protective sequestration within the mucosal immune system [25]. It is also worth wondering if regulatory T-cell presence in these same lymph nodes assists the infant early on in providing extra prophylaxis against a systemic immune response.

**Hanging in the Balance**

The variable colonization of the neonatal gut has immune responses that hinge directly on the specificity of the predominant organism. While correlations between clinical manifestations and colonization have been made, the molecular basis of these phenotypes is being elucidated. Recently, Mazmanian et al. [26] identified a unique surface polysaccharide of *B. fragilis* that induces CD4+ T-cell proliferation via novel carbohydrate MHC-II presentation by dendritic cells. Apparently the polysaccharide induces dendritic cell
maturity in order to allow for T-cell reactivity. Dendritic cells subsequently produced IL-12 which led to an increase in T-cell-generated IFNγ, the characteristic Th1 cytokine. Concurrent experiments demonstrated that CD4+ cells from germ-free mice overproduce IL-4, the cytokine associated with Th2 responses [26]. These results clearly demonstrate the potential balance achieved via competing results of epithelial responses to the gut microenvironment. It also begins to establish a molecular link to clinical observations. Recall that neonates born via CS have both decreased bacteroides colonization and increased allergic risk.

In keeping with the molecular theme of balance, two lactobacillus strains were shown to prime dendritic cells to yield regulatory T cells. These particular strains have surface glycosylation patterns that render them recognizable by DC-SIGN, a C-type lectin on the dendritic cell surface. It appears that this interaction nudges the dendritic cell toward the regulatory pathway. This effect was optimized at a bacterium to dendritic cell ratio of 1:1. A predominance of bacteria interestingly did not induce a regulatory effect. The authors speculate that this may be adaptive in the setting of bacterial overload or infection when a regulatory response would be inappropriate [27]. Again, the critical intersection of neonatal gut colonization and the interaction with epithelial and dendritic cells has another molecular manifestation. Both of these examples, while different,
demonstrate the necessary underlying machinery the neonate utilizes to assimilate the benefits of the bacterial onslaught at birth.

**Conclusion**

The fetus transitions through birth to infancy with an immune system that is readied but necessarily harnessed through regulatory mechanisms. The enormous transition from sterility to non-inflammatory colonization requires intricate adaptive responses. This is accomplished through various specific and nonspecific means, but the epithelial layer is central to the infant's ability to be colonized without harm. These interactions are central to both the immediate need to avoid infection and the long-term goal of tolerance. Recent studies have elucidated the molecular basis of the epithelial ability to provide barrier function, a non-inflammatory resting state, and protection against invasive organisms. The neonate is further assisted by the powerful exogenous immune influence via human milk. Not only does it allow proper colonization, but human milk clearly modulates neonatal excessive inflammation. Future research should be focused on better understanding the Ikk, DC-SIGN, and both the directly immune and non-immune functions of TLRs. Given the intestinal epithelial layer's open access to the environment, it seems clear that clinical intervention at this locus is inevitable. Taken in context with the widespread clinical issues of childhood allergy and inflammatory bowel disease, the gut mucosa becomes even more pertinent. The infant's acquisition of both local and systemic tolerance is complex with the reward of immunologic pearls awaiting discovery.

**References**


Discussion

Dr. Isolauri: Yesterday we talked briefly about bacterial translocation and breast milk bifidobacteria. Now you have nicely summarized the current understanding of mechanisms; how that kind of uptake could take place, and how these bacteria could be coated by IgA. Do we know anything about their fate in the common mucosal immune system after that process?

Dr. Walker: There is a study from Lausanne in Pediatrics which suggests that maternal bacteria in a mother's intestine, can be taken up across the intestine, pass
through the circulation and move to the breast where they are secreted into the breast milk [1]. I think unfortunately these studies are very early on and I don't know how to interpret them. Your group has made very interesting observations using probiotics in the latter stages of gestation showing a protective effect against allergy [2]. I think that is an area that needs to be explored, particularly in the context of programming within the intrauterine environment.

Dr. Berry: The inborn errors of metabolism sometimes provide great insight into the normal physiology. One of the things that has plagued the metabolic field for years is this mystery of *Escherichia coli* sepsis in babies with hereditary galactosemia. It only occurs in the newborn period, and appears to require exposure of the infants to lactose causing the galactose-1-phosphate levels to rise. Data are continuing to accumulate, suggesting that there is a secondary defect in glycosylation in babies with galactosemia. For example serum transferrin, the majority of molecules circulating in plasma are missing the entire chain so that the n-linked glycan with sialic acid residues is missing and as the babies are taken off lactose the assembly defect gradually disappears. We thought for some time that it was perhaps the mechanism for allowing a greater ability of *E. coli* to transfer from the lumen into the circulation of the baby and produce sepsis. The congenital defects in glycosylation where there are inherited defects in the glycosyltransferase assembling factors exist; they are terrible diseases, but they don't have *E. coli* sepsis as part of their features early on. Later on some of the patients can get protein-losing enteropathies and are susceptible to infection but it is not the *E. coli* galactosemia phenotype. One of the things that has been curious for us over the years is we have had instances in which blood cultures have been done in babies with galactosemia and evidence for *E. coli* antigens was detected in the blood but the cultures were negative, even in the absence of antibiotic exposure. Must the dendritic cell bring in the intact organism like *E. coli* or just parts of it to stimulate the proper immune response?

Dr. Walker: There is a publication by Garcea et al. [3] showing that *Bacteroides fragilis* has surface PSA on the organism. In this situation dendritic cells take up the entire organism and then the organism is modified and presented to the lymphocyte, but there are also circumstances in which secreted products from bacteria can potentially interact with pattern recognition receptors and produce the same phenomena. Macpherson and Smith [4] have done studies showing the mechanism by which commensal bacteria are taken up across M cells into dendritic cells or by dendritic cells projecting through the epithelium, and these cells are then transported to the mesenteric lymph nodes where they preferentially produce a local immune response but not a systemic response. I can't make further comments beyond those bits of information.

Dr. Prescott: I was very interested in your findings on Toll-like receptor (TLR) regulation, particularly in view of the fact that we recently observed that children who develop allergic disease actually have increased expression of TLR function to, particularly, TLR2, 3 and 4 in the neonatal period, even before significant colonization has taken place. Could you to comment on that, and also on the recent observation that breast milk appears to differentially regulate the expression of TLRs?

Dr. Walker: This is an area that is evolving. We have looked at fetal enterocytes and shown that there is an increased expression of TLR2 and 4 and that this is modulated by inflammatory circumstances. What we think is happening as part of the rationale for having increased inflammation is that immature cells express Toll receptors on the luminal surface, adult enterocytes don't; so you don't get the TLR4-mediated response that you do in the immature cell. We think that it is a maturational process which helps affect the decrease in inflammatory stimuli within the intestine. I can't comment on the allergic versus non-allergic, but I would not be surprised if Toll receptors were involved. The whole field of innate immunity in breast milk is opening up and there are lots of
factors in breast milk that we have or have not identified. There is work being done on
TLR4, TLR2 as well as CD14, suggesting again that this is something that breast milk
produces that can potentially help enhance its immune protection.

Dr. Isolauri: You mentioned that in breast milk there are some other agents that
could modify the immune response to microbes. These include fatty acids which actu-
ally engage the same TLR CD14.

Dr. Walker: That is a very important observation because initially the feeling was
that Toll receptors only interacted with microbial molecular patterns. We know now
that there are endogenous ligands including fatty acids. That is also true incidentally
in the inflammatory response in obesity.

Dr. Salminen: I would like to focus on your results from probiotics. We know from
several studies that probiotics are very strain-specific and you have used the probiotic
preparation that was defective in necrotizing enterocolitis (NEC). Are there differ-
ences in mechanistic details when different probiotic strains are compared?

Dr. Walker: We took the two strains of probiotics which were used in a clinical
study done in Taiwan [5] showing a decrease in NEC in patients at risk, that is 1,500 g
and less. We collected the strains that were described from the NIH ATTC bank and
grew them in media. Because we were dealing with the xenograph model and the skid
mouse, we were worried about putting live strains into the xenograph, so we started by
taking media from these cells and fortuitously it had an effect. It represents a response
using the same probiotics that were used clinically to prevent NEC. I totally agree with
you, you can't equate one probiotic with another because they all have different
effects and that is why we specifically used those strains. We are now considering a
multicenter trial through the neonatal network in United States, but we are not sure
which of those two probiotics is more effective. My bias would be the bifidobacteria
but I don't know. We are also not sure as to when probiotics should be introduced in
the feeding regimen of a severely premature infant, although hopefully we will soon
have some information from your institution where you have had good success in the
first introduction of feeding using probiotics.

Dr. Kunz: Like you I am very much in favor of human milk oligosaccharides.
However I don't agree in one point. Like many others you compare the prebiotic effect
of human milk oligosaccharides with prebiotic oligosaccharides from plants or modi-
ified from lactose. If the structures are compared, they are not at all the same, and with
regard to the prebiotic effect, it is most likely that human milk oligosaccharides have a
prebiotic effect but it is not shown in humans, we only have in vitro data. I agree
absolutely that there are very specific effects of human oligosaccharides on bacteria
but I am just not sure if we can call it prebiotic effects.

Dr. Walker: It depends on how prebiotics are defined. I define prebiotics as the
effect of oligosaccharides on increasing the proliferation of endogenous flora. What I
tried to show in this very new observation, in the absence of bacterial flora, this was a
cell culture situation, we used the prebiotic to reduce inflammation. To me, at least in
the preliminary observation, it appears that this might represent a primary effect as
well as a secondary effect of the oligosaccharides in the form of prebiotics. I agree with
you, milk oligosaccharides represent about 8% of the total carbohydrates in breast
milk and that is an enormous mixture of oligosaccharides, so it is very hard to isolate
out from that specific oligosaccharide. I just used as an example some of the commer-
cial oligosaccharides that presumably also exist in breast milk, although you are right,
the structure may be different.

Dr. Kunz: May You mentioned the change in sialylation to fucosylation in the
early postnatal period. Where does the fucose come from? Is it a self-regulated
process of the epithelial cell or does fucose come from breast milk or is it derived from
microorganisms?
Dr. Walker: Sonnenburg et al. [6] showed that these organisms can take complex carbohydrates and produce fucose as a substrate for the organism and presumably it is also present in the milieu. Certainly the breast milk or other forms of feeding contain fucose. I don’t think the problem is the substrate, it is the enzyme which expresses the fucose and the surface glycolipids and proteins in the glycosylation process. There are some very recent data which suggest that fucose expressed on the Toll receptor is an important determinant in glycosylation and in protection of the gut against inflammatory bowel disease in animal models. It is a very complex process and we have just began to touch the surface of it.

Dr. German: In terms of actually personalizing and bring this in essence to practice, it could be said that if a child is getting antibiotics then that would be a time to selectively avoid novel foods to avoid breaking oral tolerance. Can you imagine a time when we would actually build a tolerogenic cocktail?

Dr. Walker: As we understand more about the process, it is still again studies in progress, I would be inclined to give a child on antibiotics a probiotic because there is pretty good evidence that it protects against some of the complications.

Dr. Savilahti: Thank you for your excellent review and so much information about this development. I would like to discuss the role of prematurity in the genesis of allergic diseases. The data are rather conflicting. We completed a study about 7 years ago in which small premature babies whose birth weight was below 1,500 g were studied and again at the age of 10 years. Their allergy prevalence was one third of the control population, and the same was true for sensitization [7]. We speculated that it is probable that these very premature infants develop tolerance because we also found low levels of IgG and IgA antibodies to food antigens. So under those conditions in which there is increased permeability, there is also a possibility for tolerance development and increased permeability is always detrimental for immunological development [7, 8].

Dr. Walker: Your point is very well taken. It is much more controversial in the premature than it is in babies born by cesarean section and the use of antibiotics. You are absolutely right.

Dr. Savilahti: Another comment I would like to make is about this mechanism of probiotics because in a clinical setting we recently studied both treatment and prevention. We saw that markers of inflammation are activated during treatment. We saw an increase in CRP concentration in the serum of these infants and we also saw an increase in IL-6 concentrations and they correlated with each other [9, 10]. The probiotic was *Lactobacillus* GG, but the same happened with a combination of 4 species which we used in the prevention study [10]. Going to this hygiene hypothesis I presume that you need some kind of inflammation in the intestine.

Dr. Walker: The low grade physiologic inflammation that was discussed. The other point I want to make is I don’t think we can extrapolate from the in vitro studies that I presented, other than what we think is the possible mechanism of clinical trials. Nothing substitutes for trying a probiotic in which you think would be effective in the clinical setting in which you think it could be used.

Dr. Björkstén: We have to remember that the story is not about oral tolerance but immune modulation of relevance for respiratory allergies. It is not even about asthma, which is an inflammatory disease, but about IgE regulation and inhalant allergies. You referred to the studies by Sudo. There are actually two aspects on this. One is that gut bacteria are a prerequisite for the induction of oral tolerance. The other is that Sudo showed another type of immune regulation, that is downregulation of specific IgE antibodies by gut microbes. As you know IgE-mediated food allergy is uncommon after the age of 3 years. So I am wondering whether the discussion on mechanisms of oral tolerance has very little to do with atopic disease.
**Dr. Walker:** Good point. Again I am giving you basic observations that we carried over into the clinical setting. In most instances, for example, my using the term Th1-dominant versus Th2 for allergy versus autoimmune disease, I don’t think that holds up in humans. There is lots of evidence that the study done in Africa with schistosomiasis has a protective effect against the expression of allergy, not allergy per se, but allergic symptoms. It is a much more complex process than we think.

**Dr. Björkstén:** In Africa immune deviation is said to be due to parasites, but we have published almost identical cytokine immune responses in Estonian as compared to Swedish children, and Estonians don’t have parasites that influence the IgE antibody formation. I would suggest that it is conceivable that what you see in Africa may not only be due to parasites. They may also have a totally different gut flora. This has not been looked at. As I said we have excluded parasites in Estonia.

**Dr. Walker:** Another good point. There are lots of clinical questions that need to be resolved. In the interest of time, I was more or less trying to tweak your interest by giving you an overview in a somewhat simplistic fashion.

**References**
