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Volume 635

GI Microbiota and Regulation of the Immune System

Edited by
Gary B. Huffnagle
Mairi C. Noverr

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PREFACE

The idea that the microbial communities within the GI tract have a profound influence on general human health actually originated with Russian scientist Elie Metchnikov at the turn of the last century. Also known as the "father of immunology", Metchnikov believed that putrefactive bacteria in the gut were responsible for enhancing the aging process. He theorized that ingestion of healthy bacteria found in fermented foods could counteract toxic bacteria and was the key to good health. His theories concerning good bacteria and health can be found in his treatise "The Prolongation of Life: Optimistic Studies". These writings prompted Japanese scientist Minoru Shirota to begin investigation of how fermentative bacteria improve health. He succeeded in isolating a strain of Lactobacillus that could survive passage through the intestine, while promoting a healthy balance of microbes. The "Shirota strain" is still used today in the fermented beverage Yakult. It is clear from a commercial standpoint that these ideas have inspired the development of a probiotic industry, which has expanded greatly in the U.S. over the past 5-10 years.

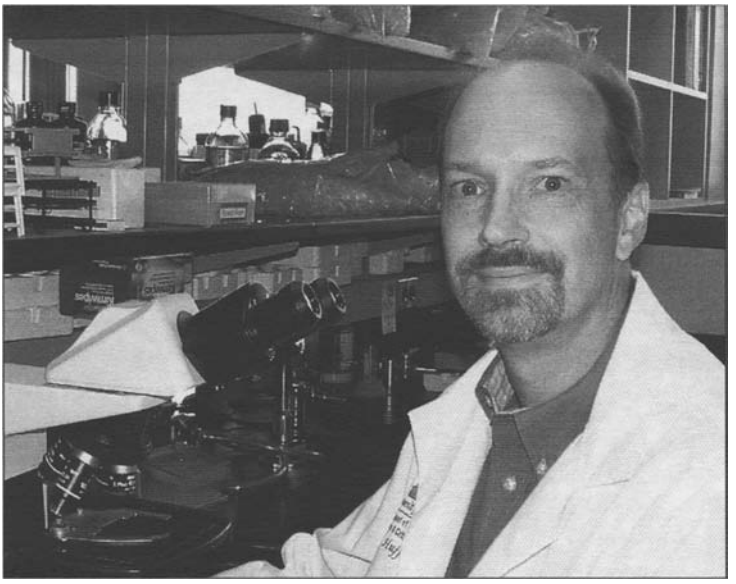
Likewise, scientific studies investigating the microbiota and the immune system have increased significantly in recent years. This increase in research is also due to advances in technologies that enable the investigation of large microbial communities, a resurgence in gnotobiotic animal research, and improved methods for molecular analysis of probiotic bacterial species. Our interest in this area stems from our laboratory observations indicating that antibiotics and fungi can skew microbiota composition and systemic immune responses. Our initial base of references upon which to develop further hypotheses concerning the mechanisms involved in microbiota regulation of immune responses was limited. However, in presenting the research at national scientific meetings and at universities across the country, the feedback and interest were overwhelming. It became clear that a book dedicated to current trends in investigating the GI microbiota was warranted. Dissection of the relationship between the microbiota and the immune system is currently being approached from a variety of angles that we have sought to incorporate into this book.

This book opens with two general reference chapters, which provide an overview of current knowledge of gastrointestinal immunology and the commensal microbiology of the gut. Next are two chapters dedicated to current methodologies

used to investigate the microbiota and host: molecular analysis of microbial diversity and gnotobiotic research. Both positive and negative interactions between the microbiota and the immune system can take place in the gut, with chapters dedicated to probiotics and intestinal diseases associated with unhealthy microbiota. Environmental factors play an enormous role in shaping the microbiota composition. Host, microbial, and dietary factors take part in a complex interplay, which provides many distinct and diverse research subjects. We have included a chapter discussing diet, functional foods, and prebiotics, which are dietary supplements used to specifically enhance the growth of beneficial members of the microbiota. Several laboratories are investigating how the different members of the microbiota communicate with each other and with the immune system. A chapter reviewing how bacteria sense and respond to signaling compounds in the gut environment provides insight into the signal transduction pathways that mediate interactions between the host and microbiota. A highly detailed and well-investigated model of bacterial-host symbiosis provides an immense amount of background and insight for the developing field of host-microbiota studies. We have included a chapter reviewing the unique interactions that take place in a non-mammalian system, the Squid-Vibrio model. Finally, we close the book with two chapters outlining current hypotheses concerned with redefining our understanding of the relationship between microbes, disease, and the basic mechanisms of immune system function.

Gary Huffnagle, PhD and Mairi C. Noverr, PhD

ABOUT THE EDITORS...



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CHAPTER 1

Overview of Gut Immunology

Katie Lynn Mason, Gary B. Huffnagle, Mairi C. Noverr and John Y. Kao*

Abstract

The gastrointestinal tract (GI tract) plays dual roles in human physiology: digestion and uptake of nutrients and the more daunting task of maintaining immune homeostasis (protecting the body from potentially harmful microbes, while inducing tolerogenic responses to innocuous food, commensals and self-antigens). The unique architecture of the GI tract facilitates both of these functions; multiple levels of infolding results in an immense overall surface area that allows maximal nutrient absorption while housing the largest number of immune cells in the body. This review will focus on how mucosal immune responses generated in the GI tract are organized and controlled. The gastro-intestinal associated lymphoid tissue (GALT), which is composed of discrete inductive and effectors sites, is able to discriminate between harmful and harmless antigens while maintaining homeostasis. Inductive sites are organized int o specialized aggregations of lymphoid follicles called Peyer's patches (PP), while effector sites are more diffusely dispersed. The separation of these sites serves to limit and control immune responses. In addition to its distinct architecture, the GI tract has specialized immune cells that aid in promoting a tolerogenic response to orally introduced antigens, (e.g. subsets of dendritic cells (DCs) and regulatory T-cells (T_R)). Secretory IgA (sIgA), which is produced in appreciable quantities at mucosal surfaces, also promotes an anti-inflammatory environment by neutralizing immune stimulatory antigens. The mechanisms of induction tolerance are currently poorly understood; however, this tolerant environment limits potentially damaging inflammatory responses to inappropriate stimuli.

Introduction: Tolerance vs. Inflammation

The GI tract has the difficult task of protecting the body from potentially pathogenic organisms (PPOs) while at the same time providing an environment tolerant to commensal microbes, dietary antigens, and self-antigens. Mucosal surfaces are the site of entry for many pathogens; however these regions of high susceptibility are also constantly mounting immune responses, whether inflammatory or tolerogenic, to the numerous antigens that come into contact with the mucosa. Because the majority of antigens that come in contact with mucosal surfaces are nonharmful, the majority of immune responses elicited in these regions induce tolerance. Systemic nonresponsiveness to antigens that are introduced orally is a phenomenon known as oral tolerance.

There are multiple mechanisms involved in induction of tolerance in the GI tract (Table 1). Mechanisms broadly fall into two categories: antigenic ignorance and active tolerance. Antigenic ignorance involves preventing antigens and microbes from gaining access to the immunoreactive areas within the GI tract. Active tolerance involves induction of antigen specific and nonspecific anti-inflammatory responses and/or deletion of reactive immune cells. In studies of oral tolerance in mice, low-dose oral antigens led to an active suppression of the gut immune response while high-dose feeding regimens led to anergy.[1] Oral tolerance is typically characterized by the

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Table 1. Mechanisms of immune tolerance in the GI tract. Many of the factors within the gastrointestinal tract sway immune responses towards a tolerant environment

	Mechanisms of Immune Tolerance in the GI Tract
•	Production of secretory IgA (sIgA)
•	Preference for Th2 responses
•	Unique anatomical design
•	Presence of specialized immune cells
•	Specialized adhesion molecules and chemokine receptors
•	Effects of the indigenous microbiota

suppression of the systemic Th1 response to antigens and elevated levels of IL-10, TGF- β , and antigen-specific sIgA at the mucosal surface. The Th2 response also promotes the induction of tolerance in the gut.[2] Production of IL-4 and IL-5 during Th2 responses acts synergistically to enhance IgA production. These cytokines also act to further inhibit the Th1 response.

Several factors can prevent induction of oral tolerance as demonstrated in animal models. Co-inoculation of antigen along with an adjuvant, such as cholera toxin or saponin, will provoke a robust immune response.[3,4] In addition, deletion of the indigenous microbiota using either germfree animals or broad spectrum antibiotics prevented induction of oral tolerance.[5,6] While tolerance may be the default response, the GI tract must also protect against PPOs, which include both "professional" and "opportunistic" pathogens. "Professional" or toxin-producing pathogens are acquired from exogenous sources, causing harm to the host (e.g., *E. coli* O157:H7). "Opportunistic" pathogens are often normal members of the microbiota but can cause harm to the immune suppressed host often due to overgrowth (e.g., *Candida albicans*). Both innate and adaptive responses collaborate in controlling infections by PPOs and preventing systemic dissemination via the GI tract to the bloodstream. The dynamic interactions that occur in the normal gut create an environment that is tolerant to dietary antigens, protective against potential pathogens, and able to maintain gut immune homeostasis.

Gastrointestinal Tract Architecture

The architecture of the gastrointestinal tract is designed to facilitate the dual roles handled by the organ: nutrient uptake and defense against PPOs.[2] The vast surface area of the GI tract (≈200 m^2) is the result of several levels of invagination at the tissue (Kerkring folds), cellular (villi) and membrane levels (microvilli). At the cellular level, villi are lined with intestinal epithelial cells (IECs) that have absorptive microvilli to optimize the absorption of nutrients released during digestion. The tips of these microvilli form the filamentous brush border glycocalyx (FBBG) that is composed of a layer of membrane-anchored glycoproteins, which allow nutrients to cross, while restricting entry of whole bacteria or large molecules.[7] To block entry and/or reduce damage caused by PPOs, the GI tract has an effective repertoire of defense mechanisms. The protective defenses of the GI tract include physical barriers, antimicrobial compounds and specialized immune responses.[8] The luminal contents are separated from underlying lymphoid tissue by the intestinal epithelium, which serves as a restrictive physical barrier held together by intercellular tight junctions that can block extremely small molecules (>2 k-Da).[9] Antimicrobial peptides, mucins and trefoil peptides also act to restrict pathogen access to mucosal surfaces.[10,11] In addition to these host factors, the indigenous microbiota plays an active role in not only preventing establishment of PPOs via competitive exclusion, but also by influencing the gut immune responses (discussed later).

The architecture of the gastrointestinal associated lymphoid tissue (GALT) is designed to limit and control immune responses via separation of inductive and effector sites. Inductive sites consist of organized aggregation of lymphoid follicles and include the Peyer's patches (PP) and mesenteric

lymph nodes (MLNs).[12] PPs are typically found within the distal ileum of the small intestine, where the microbiota is more abundant and diverse. However, recent reports have shown that PP may not be restricted to the distal ileum due to the discovery of lymphoid aggregates that have PP appearance in other locations throughout the GI tract as well.[13] Mature PPs are macroscopically visible domes and have an organizational structure similar to lymph nodes. The subepithelial dome (SED) of PPs is made up of large B-cell follicles with intervening T-cell areas that work to collect antigen from epithelial surfaces within the GI tract. The SED is separated from the lumen by the follicle associated epithelium (FAE), which is a monolayer composed of columnar epithelial cells that have a less distinct brush border, fewer digestive enzymes, and high numbers of immune cells (for a review see ref. 14). One distinct feature of this specialized monolayer is the presence of M-cells, so named for their "microfold" appearance. Formation and development of full-sized M-cells requires stimulation from mature B-lymphocytes, predominantly via the expression of LTα1β2.[15] The filamentous brush border glycocalyx, as well as the typical mucus layer of the GI tract, is missing from the apical surface of these cells.[12] M-cells transport antigens to specialized APCs within the underlying SED, through vesicular transport (Fig. 1).[7]

Mesenteric lymph nodes (MLNs) are distinct from other specialized tissues in the GALT due to their normal development in the absence of growth factors that are necessary for development of PP, M-cells and even other peripheral lymph nodes. They are also larger than all other lymph nodes in the body.[12] However, within germfree mice the MLN are much smaller, but still functional, due to a lack of stimulation by the normal microbiota.[16] Lymphocyte migration to lymph nodes is mediated by lymphocyte cell surface receptors binding to ligands on high endothelial venules (HEV). Lymphocte L-selectin and α4β7 integrin expression are required for homing to the MLNs. The α4β7 integrin is known for its role in directing lymphocytes to mucosal tissues by binding to mucosal addressin cell adhesion molecule-1 (MAdCAM-1) on HEV in both the MLNs and PP. It has also been shown that L-selectin guides lymphocytes to the peripheral tissues.[17] Because most circulating

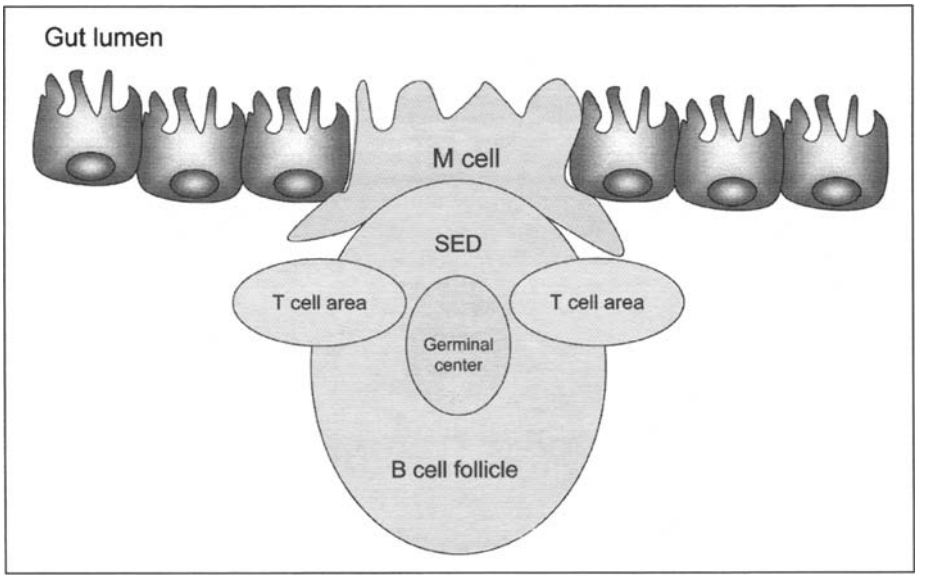

Figure 1. M-cell and gut-associated lymphoid tissue architecture. This diagram shows the location of specialized M-cells within the follicle associated epithelium, overlying GALT. Directly beneath the M-cell is the sub-epithelial dome that is rich in dendritic cells while the area underneath the SED is lymphoid tissue dense in B- and T-cells. One proposed mechanism of antigen recognition within the GI tract is that antigen enters through the M-cells to be presented to the underlying B- and T-cells by dendritic cells from the SED.

lymphocytes express both L-selectin and $\alpha 4\beta 7$ integrin, immune interactions between the periphery and GALT may play a role in directing responses between the gut and the entire host.

Although the GALT and MLNs are the primary tissues involved in GI immunity, intestinal epithelial cells also play a unique and underappreciated role in regulating immune responses. These epithelial cells can secrete immune signaling molecules (cytokines, chemokines and eicosanoids) both constitutively and after stimulation from PPOs, which helps direct both innate and adaptive responses (for a review see ref. 18). Conditioning of dendritic cells (DCs) in close proximity to the IECs occurs in the absence of inflammatory signals or infection. Exposure of DCs to epithelial cells results in IL-10 production, but not IL-12, indicating that IECs in the steady-state induce tolerogenic responses.[19] IECs also participate in initiating adaptive immune responses in the gut by transporting luminal antigens to underlying immune cells for presentation by professional antigen presenting cells or can present antigen themselves. IECs are polarized so that antigen is processed apically and presentation to T-cells via class II molecules can only occur at the basolateral surface.[20] IECs are MHC class-II positive, but typically do not express the costimulatory molecules necessary to activate T-cells.[21] Also, CD4+ T-cells are not commonly found in the lamina propria and those T-cells that are present do not migrate out of the gut.[22] Despite these factors, IECs could play a role in maintaining the T-cells that have been primed previously and then localized to the lamina propria. Due to these factors, IECs can be seen as an exception to the normal routes previously proposed for induction of an immune response.

Components of the Gut Immune Response

Dendritic Cells

The decision between inducing mucosal tolerance or inflammation is made by immune cells situated at several distinct locations of the intestinal tract. The decision depends both on inherent properties of the antigen and is directed by professional APCs (e.g. intestinal DCs). Several specialized subsets of DCs can be found within the GI mucosa, both inductive and effector sites. Even the villus mucosa has specialized DCs that are not well studied, but may play a role in antigen uptake. The percentage of different subsets can differ drastically between the different lymphoid tissues, pointing towards unique specialized roles for different lymphoid tissues and their associated APCs. The presence of many different DC subsets complicates studies of understanding how responses in the GALT are coordinated. Different cell types that are nearby, antigen itself, or any number of other factors could influence the behavior and activation of DCs. It is not known whether specific subsets of DCs are functionally restricted to drive specific types of responses, or whether DC subsets exhibit plasticity. Determining the role of each of these subsets in GI immunology is daunting, but DCs are the major gatekeepers for directing the multiple innate and adaptive immune responses that occur within the GI tract.[23]

In the murine GALT, three distinct subsets of DCs have been identified based on their differential expression of specific cell surface markers and their characteristic localization.[24] All subsets express CD11c and major histocompatibility complex (MHC) class II, but differ in their expression of CD8α and CD11b. Myeloid DCs (CD11c+ CD11b+) are localized in the SED of PPs under the follicle associated epithelium, where they are positioned to receive luminal antigens transported by M-cells. Interfollicular regions of PPs are populated by CD11c+ CD8α+ lymphoid or plasmacytoid DCs. A third subset of "double-negative" (CD8α–CD11b–) DC has also been reported to populate both locations. In addition, DCs within the PP that are CD11c low are also present.[25] LP DCs are not well-studied, but subsets in this area may be similar to those found in the PP.[26]

Whether an immune response is directed towards inflammation or tolerance is influenced by immune signaling molecules originating from DCs and T-cells. An interesting characteristic of PP DCs is that after activation by ligation of RANK, the DCs produce IL-10 which promotes an anti-inflammatory response; however, the same activation signals induce splenic DCs to produce pro-inflammatory IL-12.[27] This drastic difference in response, based upon location of the lymphoid

tissues, shows how antigen is handled differently by the GI immune response compared to a systemic response. The specialized DCs in the PP also polarize antigen specific T-cells to produce IL-10, promoting regulatory T-cell (T_R) expansion, activation and/or differentiation.[24] Recent evidence points towards CD11b+ subsets within the PP playing a tolerogenic role via low IL-12 and high IL-10 secretion. These DCs lead to T-cell activation and further IL-10 secretion.[24] However, the CD8α+ and CD11b− subsets of DCs within the PP have been found to produce high levels of IL-12 that can lead to an inflammatory response under the appropriate conditions.[28]

Within the LP there are also unique subsets of DCs that are similar to those found in the PP, including CD11c+ CD11b−. Although these have not been studied in as much depth as those within the PP, their role has been hypothesized to include anti-inflammatory responses. While CD11c+ CD11b− DCs are found within the LP; within the terminal ileum, the dominant subset of DCs has been found to be the CD11c+ CD11b+ DCs.[29] A characteristic marker of LP DCs is the expression of integrin α chain CD103 (αE integrin).[30] CD103 is expressed by subsets of DCs, usually from the mucosal tissues, as well as CD4+ and CD8+ T-cells.[31,32] CD103 was suggested to play a role in T-cell homing to the intestine by pairing with β7 integrin, which enables binding to E-cadherin (expressed on intestinal epithelial cells).[33] However, adoptive transfer of either wildtype or CD103−/− CD4+ CD25+ T_R cells was able to suppress colitis in mice. Adoptive transfer of wildtype naïve CD4+ T-cells into CD103−/− mice results in colitis.[34] Therefore, T-cell trafficking to the gut is not regulated by CD103 expression. Surprisingly, CD103 expression does correlate with differential DC function.[34] Adoptive transfer of wildtype CD4+ CD25+ T_R are unable to suppress colitis in CD103−/− recipient mice. Examination of wildtype and CD103−/− DC revealed that only wildtype DCs promote upregulation of CCR9, which allows homing to the intestine by binding to CCL25. In addition, CD103−/− DCs promote pro-inflammatory IFNγ production by T-cells.[34] Despite the lack of knowledge about other specific subsets of DCs within the LP, CD103 expression seems to play a unique role regulating tolerance via effects on T_R.

Microbial Discrimination: TLRs and Danger Signals

Dendritic cells have the ability to detect groups of microbes nonspecifically by recognizing pathogen associated molecular patterns (PAMP). PAMPs bind to pathogen recognition receptors (PRR) on the surface of DCs, which result in downstream activation or inhibition of pathways involved in inflammation. The most famous group of PRR are the toll-like receptors (TLR), which form homo- and hetero-dimers. To date, 13 TLRs have been described, which bind to a wide variety of both microbial and host compounds. For example, TLR4 binds to LPS from gram negative bacteria, TLR5 binds to flagellin and TLR2/6 binds to fungal zymosan (for a review see ref. 35).

One dilemma the GI tract must face is the use of TLRs in recognizing potentially infectious microbes. Discrimination between pathogens and commensals is complicated by the fact that both groups of microbes possess the same PAMPs. Several mechanisms may be involved in determining whether a microbe signals a pro-inflammatory response via TLRs. It has been hypothesized that commensals are sequestered on the epithelial surface within the gut, preventing activation of TLRs while pathogenic bacteria express virulence factors that enable attachment to and penetration of the epithelium and gain access to underlying DCs.[36] However, commensal bacteria are recognized under normal steady state conditions by TLRs in the GI tract. In fact TLR signaling aids in maintenance of GI tract homeostasis, as well as providing protection from injury by pathogenic bacteria.[37] This discovery has complicated our understanding of the role of TLRs in pathogen discrimination.

An alternative interpretation of the function and activity of TLR has been championed by Polly Matzinger as an extension of the danger model (for a review see ref. 38). She proposes that these receptors actually sense and respond to hydrophobic danger signals termed hyppos (hydrophobic portions), coming from damaged host or microbial sources and stimulate inflammatory responses.[39] TLR4 binds to the membrane buried portion of LPS, a cytosolic fusion protein of

respiratory syncytial virus and also host components such as heat shock proteins and hyaluronan, which are released upon host cell or microbial cell damage.

Another mechanism that may be involved in determining how and whether TLR signaling generates a pro-inflammatory response is the discovery of multiple types of negative regulators (for a review see ref. 40). These include extracellular decoy receptors, intracellular inhibitors and membrane-bound suppressors. In addition, inhibition can be mediated by degradation of TLRs and TLR-induced apoptosis. Soluble decoy TLR4 and TLR2 act as nonsignaling extracellular sinks for TLR agonists. Intracellular negative regulators can function at various stages of the TLR signaling cascade but work principally via actions on MyD88. Negative regulators that function at this stage include MyD88s (a truncated form of MyD88),[41] IRF,[42] SOCS,[43] NOD2,[44] PI3K,[45] TOLLIP[46] and A20.[47] Transmembrane protein regulators act by sequestering adaptor proteins (ST2)[48] and transcription factors (TRAILR),[49] or by interfering with the binding of TLR agonists to their respective TLRs (SIGIRR and RP105).[50,51] Reduction of TLR expression occurs by ubiquitination of TLRs (TRIAD3A), which targets them for proteasomal degradation.[52] Excessive TLR activation could lead to caspase-dependent (through the death domain of MyD88) and caspase-independent apoptosis.[53,54] Over exuberant and destructive inflammation occurs in knockout mice deficient in several of these negative regulators (SIGIRR, NOD2), demonstrating the importance of control of these pathways.[50,55] It remains to be determined whether specific groups of microbes (nonpathogens) prevent TLR signaling by activating a negative regulation pathway.

T-Cells

Mucosal DCs play a direct role in activation, expansion and induction of T-cells in the GALT that control and maintain tolerance. The major T-cell type involved in regulation of tolerogenic responses is the regulatory T-cell (T_R). Formerly known as suppressor T-cells, several subsets of T_R have been identified on the basis of cellular markers and mode of suppression. Both antigen nonspecific (natural or steady-state) and antigen-specific (induced or adaptive) T_R are found within the GALT. In addition, recent studies have pointed to intraepithelial lymphocytes (IEL) playing a role in promoting tolerance in the GI tract.

Natural T_R are generated in the thymus and constitutively express CD25 (IL-2 receptor), CTLA-4 (cytotoxic T-lymphocyte antigen-4) and GITR (glucocorticoid induced tumor necrosis factor receptor) and the transcription factor Foxp3 (forkhead box protein 3) (for a review see ref. 56). This CD4+ CD25+ subset plays a critical role in maintaining homeostasis within the body by recognizing self-antigens and preventing an inflammatory response to chronic stimuli via bystander effect.[57] Natural T_R are not all CD25+, with CD25 expression level influenced by activation state. Another complicating factor in studying T_R is that activated CD4+ T-cells also express CD25, CTLA-4 and GITR, which hinders the use of these markers in identifying T_R. In healthy mice, the percentage of T_R in the CD4+ CD25+ population is around 90%, however this percentage may change under inflammatory responses where activated T-cells could pollute the normal CD4+ CD25+ population.[58] However, Foxp3 expression appears to be limited to T_R and its expression is associated with suppressive activity.[59,60] Foxp3 is difficult detect in vivo because of its nuclear location, which has hindered the study of the biology of Foxp3-expressing cells. Recently, investigators constructed a transgenic murine model to monitor Foxp3 expression using a bicistronic reporter expressing a red fluorescent protein which was knocked into the endogenous Foxp3 locus.[61] Foxp3 is predominantly expressed in CD4+ CD25+ peripheral T-cells, but is also found in a subset of CD4+ CD25– peripheral T-cells. TGF-β induces Foxp3 expression along with suppressive function in CD4+ T-cells.[61] This Foxp3 reporter system should aid in furthering the study of regulation and function of T_R. While Foxp3 is a more definitive marker for functionally suppressive T_R, CD25+ T_R have been more well-studied in the context of gut tolerance. Adoptive transfer of CD25+ T_R in a murine model of colitis was effective at suppressing both induction of disease and curing established disease.[62,63] In addition, CD25+ T_R are also involved in mediating oral tolerance, although CD8+ suppressor T-cells may play a role as well.[64,65]

Adaptive T_R subsets that have been described in terms of intestinal immunity include Th3 cells and Tr1 cells. Th3 cells are a subset of the CD4+ T_R population and are seen after the induction of oral tolerance.[66] Low doses of oral antigen induce Th3 cells that traffic to the MLNs in mice.[67] They act in a suppressive manner under the influence of TGF-β, however the lack of a specific marker for Th3 populations has complicated further study to elucidate their individual role in oral tolerance. Due to their production of TGF-β, they can influence the production of IgA. Tr1 cells are also CD4+ T-cells that produce IL-10 and play a role in suppressing an inflammatory response. Tr1 cells are also a T_R population but are Foxp3 negative.[68] Although they have not been proven to play a role in vivo, Tr1 cells can produce bystander suppression of experimental colitis in mice and their secretion of IL-10 is hypothesized to minimize damage to the host during a chronic infection.[69,70]

Intraepithelial lymphocytes (IEL) represent an unusual T-cell population that are dispersed throughout the GI tract and are found in close proximity to the epithelial layer, above the basement membrane. IELs are believed to mature extra-thymically within the LP in areas called cryptopatches.[71] IELs include CD8αα+ and TCRγδ T-cells and both types have been shown to play a role in suppression responses in mice. Adoptive transfer of either CD8αα+ can prevent induction of colitis.[72] Injection of γδ T-cells from mice previously tolerized mice can transfer antigen specific oral tolerance to naïve recipient mice; conversely depletion of γδ T-cells prevents the induction of oral tolerance.[73] Further studies of these specialized T-cells are needed to help determine the mechanism of tolerance induction.

B-Cells and Antibody

Secretory IgA (sIgA) is the major immunoglobulin isotype found in most mucosal surfaces, with 80% of all plasma B-cells located in the GI tract and 3 g of sIgA are produced per day in humans.[74,75] This polypeptide complex is made of two IgA monomers with a connecting J chain and secretory component (described below). It plays a crucial role as an anti-inflammatory component within the mucosa due to its ability to bind innocuous antigens and inability to activate complement. However, IgA found in serum is primarily monomeric (mIgA) and originates primarily from within the bone marrow.[76] Surprisingly, mIgA within sera interacts with FcαR as an inflammatory immunoglobulin as opposed to its anti-inflammatory properties within the mucosa.[77] IgA monomers are composed of two heavy and two light chains like other immunoglobulins; however, IgA is unique in its ability to further polymerize (pIgA). A 15 kDa polypeptide named the J chain, as well as disulfide bridges, helps two monomer units of IgA stabilize in an end-to-end configuration. Both the J chain and IgA are synthesized in plasma cells with the J chain initiating polymerization of the IgA monomers.[78-80] Within the GI mucosa, IgA exists predominantly as dimers, but trimers and tetramers form as well.[81]

The ability of the GI epithelium to transport pIgA across its epithelial barrier into mucosal secretions is one of the primary mechanisms utilized to protect mucosal surfaces from pathogens. The transport of pIgA relies upon the interaction of a 110 kDa transmembrane glycoprotein, named pIgR, which is expressed on the basolateral surfaces of mucosal epithelial cells. pIgR is internalized by endocytosis into basolateral endosomes and then sorted for transcytosis across the epithelial cell.[82] Once the receptor reaches the apical membrane, it is cleaved by a serine protease at the junction between the extracellular domain and the membrane-spanning region. The extracellular part of the receptor that is released by this protease is now called the secretory component and is included in mucosal secretions.[83] The secretory component acts to stabilize pIgA and to provide some resistance to further protease activity within secretions and this molecule is now termed sIgA.[84] Secretory component also has glycosylated residues that further stabilize sIgA by anchoring the immunoglobulin within the mucus to further its role in mediating immune responses within the gut.[85] Secretory component can also act on its own within mucus as a scavenger towards enteric pathogens.[86] This transport system is effective in shuttling antigens that get past the epithelial layer back into the lumen, preventing antigen access to mucosal immune areas.

The importance of IgA-mediated protection is widely accepted, but IgA also is intrinsic in maintaining gut homeostasis and tolerance. IgA is spontaneously induced by the presence of commensals in the intestine. Normal mice are systemically ignorant of commensal microbes; however, upon intravenous injection of these organisms, IgG responses are induced.[87] Some of this protection due to IgA; mice deficient in IgA are more likely to have spontaneous priming of a systemic response to commensals.[87] Induction of specific IgA is dependent on T-cell help; however, in T-cell deficient mice, IgA is not completely ablated. IgA is only partly dependent on T-cell help and in particular the responses to the commensal intestinal microbiota are more T-independent that responses to adjuvants (cholera toxin).[88] This can be explained by the fact that protection against the microbiota would require a broad spectrum IgA response, whereas production of neutralizing IgA for bacterial toxins would require a highly specific high-affinity IgA that would require T-cell help. This same phenomenon may facilitate the seeming plasticity of the IgA profile in the intestine, which is continually responsive to changes in the microbiota.[87]

In humans IgA deficiency is common and is associated with increased susceptibility to gastro-intestinal infections, ulcerative colitis, and IBD.[89,90] However, it has been also proposed that a secondary and equally important role of IgA is in regulation of the gut microbiota. For example, in mice deficient in IgA production, preferential expansion of the segmented filamentous bacteria (SFB) are observed.[91] SFB strongly attach to the gut epithelium and are the major bacterial species detected in gut epithelial cell samples of these mice. Further, IgA deficiency in humans is associated with increased numbers of *E. coli* strains with potentially pro-inflammatory properties such as S fimbriae and haemolysin.[92] These alterations in the microbiota may be responsible for pathological effects in the GALT.

Coordination of the Gut Immune Response

Antigen Route of Entry

The pathway for the induction of an intestinal immune response is still under debate due to the complexity of the system and the difficulty in studying a process with a high number of variables (Fig. 2). There are several possible routes of antigen uptake and presentation in inductive sites. In PP, M-cells in the FAE can transfer antigen to underlying DCs. These DCs can either present antigen to T-cells within the PP or can enter the draining lymph and traffic to the MLNs. Another route of antigen entry involves uptake through the epithelium of the LP and delivery to underlying DCs or other enterocytes that express MHC class II, which can travel to the MLNs. A third route involves direct antigenic sampling of the intestinal lumen by DCs within the lamina propria, which express tight junction proteins allowing for extension of dendrites between epithelial cells.[93] The chemokine receptor CX3CR1 (fractalkine receptor), which is expressed on LP DCs throughout the small and large intestine, was found to be involved in trans-epithelial dendrite extension for luminal antigen sampling in mice.[94] This same group also determined that CX3CR1 aids in the clearance of invasive pathogens by DCs. The relative importance of each of these routes of entry remains to be determined.

DC Trafficking

DCs are the primary antigen presenting cell type in the GALT and travel within and between inductive (both PP and the MLNs) and effector areas (LP) based on receptor ligand interactions. Both steady-state and inflammatory migration occurs in the GALT. Chemokine receptors have been implicated in recruitment and guidance of DCs to different locations within the GI tract. CCR6 aids in recruitment of PP DCs to the SED and is necessary for activation of T-cells in the presence of pathogens.[95] CCR2 is involved in DC recruitment to the LP, while CCR7 aids in recruitment to the MLNs.[96] It is believed that migration from the intestine to the MLNs is crucial in development of tolerance. As evidence of this, mice deficient in CCR7 are unable to be tolerized to oral antigen.[97] Results from studies investigating DC trafficking in the GI mucosa indicate that most DCs that enter the MLNs actually originate from the LP.[98] Steady-state trafficking of conditioned DCs from the intestine to the MLNs is thought to be intrinsic in maintenance of

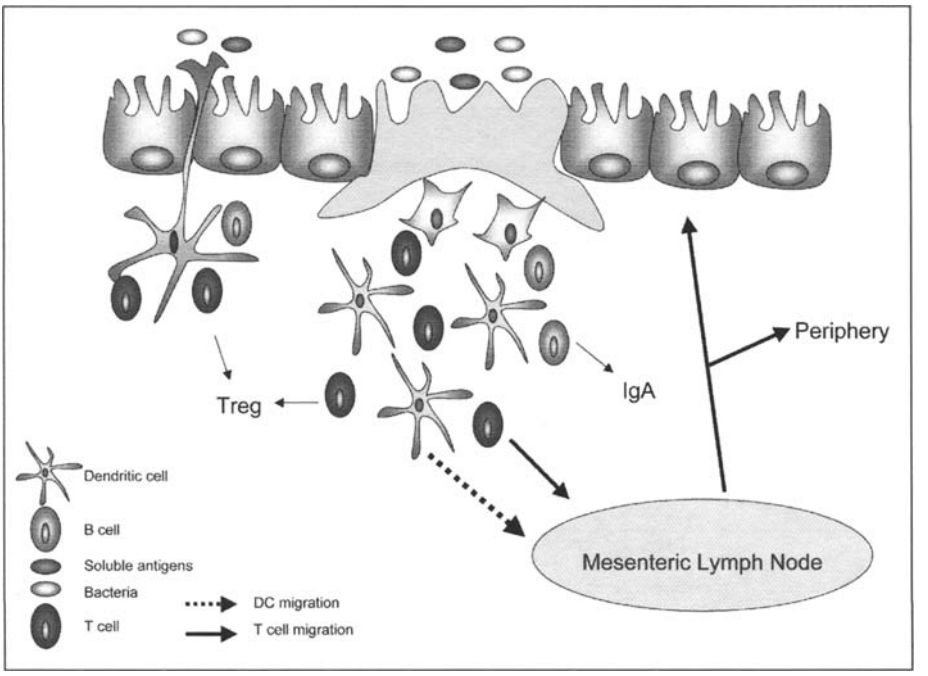

Figure 2. Routes of Antigen Recognition in the GI tract. Several potential routes of entry for orally administered antigen in the GI tract are outlined in this figure. Antigens may enter through M-cells in the follicle associated epithelium where local DCs present directly to T-cells within the PP. Antigens or antigen-loaded DC may also directly enter the draining lymph and travel away from the PP towards the MLN for T-cell recognition. In addition, antigen may also access the MLN's by traveling through the epithelium of the villus lamina propria. Alternatively, these antigens may be taken up by the enterocytes that express MHC class II molecules enabling them to prime T-cells. Another possible point of entry for antigen may be through the lamina propria with direct access to the blood stream for travel to the peripheral lymph nodes where the antigens can interact with T-cells. Finally, DCs within the lamina propria can extend out into the lumen to sample antigens directly.

tolerance to innocuous antigens via inactivation of autoreactive T-cells, promoting differentiation of naïve T-cells towards a regulatory phenotype, or activation and expansion of pre-existing T_R.

Effector Cell Interactions

Antigen-loaded conditions DCs either prime cells locally travel or traffic to other areas of the GALT. In the PP, DCs and T-cells deliver cellular signals such as TGF-β and IL-10 which activate B-cells to undergo immunoglobulin class switching from IgM to IgA within the PP to aid in inducing an anti-inflammatory response.[99] Macpherson et al found that purified DCs loaded with bacteria could directly stimulate IgA production by B-cells.[100] The primed lymphocytes leave the PP and travel through the draining lymphatics to the MLNs where they undergo further differentiation before continuing on to the bloodstream via the thoracic duct and finally enter effector sites such as the LP.[12] These primed and differentiated effector T-cells traffic to the LP via loss of L-selectin and up-regulation expression of $\alpha_4\beta_7$ integrin and CCR9. The $\alpha_4\beta_7$ integrin interacts with mucosal addressin cell-adhesion molecule 1 (MAdCAM1) that is expressed by mucosal surfaces within the LP, while CCR9 binds to CCL25 expressed on villus crypt cells.[101]

The LP is the major site of effector responses in the GALT and is populated with conditioned DCs, primed T- and B-cells, IELs and macrophages. Upon arrival in the LP, B-cell blasts mature

into IgA producing plasma cells. Several subtypes of T_R are suggested to reside within the LP; isolated LP CD4+ T-cells exhibit hyporesponsiveness to antigen via production of large amounts of TGF-β, IL-4 and IL-10.[102-104] CD8+ T-cells migrate, for the most part, from the LP to the epithelium. However, a subset also remain in the LP, which have cytotoxic T-lymphocyte activity.[105] It is unknown whether these T-cells are effector T-cells aiding in production of IgA or are simply memory cells. A study conducted by Masopust et al found that antigen specific memory CD8+ and CD4+ T-cells both accumulate in the intestinal mucosa.[106] The IELs in the LP exhibit tolerogenic activity separate from T_R. IELs can also be stimulated by a variety of pathogens and may play a role in antimicrobial defenses in the LP.[107] Macrophages in the LP are proposed to function as antibacterial guards, phagocytosing and killing any microbes that penetrate the LP epithelium (for a review see ref. 108).

Importance of the GI Microbiota

The importance of the GI microbiota in mounting host immunological responses has been reinforced through work with germfree (sterile) and gnotobiotic (defined microbiota) animal models. These models have greatly simplified studies of microbiota interactions with the host. Altered-Schaedler Flora (ASF) mice are the most complex gnotobiotic model currently available. ASF mice are intestinally colonized with eight bacterial species including two lactobacillus species.[109] These eight strains were chosen due to their stability over generations as well as the return of wild type characteristics once germfree mice are colonized with the ASF microbes.[110] Germfree mice typically have enlarged cecums and are highly susceptible to opportunistic pathogens. Conventionalization with murine microbiota or ASF strains reverses these adverse characteristics. Germfree mice have additional systemic abnormalities such as smaller lungs and hearts and lowered cardiac output.[16]

The indigenous microbiota of the gastrointestinal tract has been implicated in playing a role in many physiological processes, ranging from host metabolism to immune education. Recently, evidence has been mounting for a role in changes in the microbiota contributing to development of systemic and localized inflammatory diseases. Atopic dermatitis, eczema, food allergy, inflammatory bowel disease (IBD), pouchitis and vaginitis have all been linked to perturbations of the normal microbiota.[111-117] In humans, studies are limited to correlative epidemiological studies, which have been hampered by a lack of definitive and reproducible methods to monitor the microbiota. Therefore further work using germfree, mono-associated and gnotobiotic animals will help define which species are positively and negatively associated with disease so that we can begin to dissect the mechanisms mediating these effects on the host.

Summary

The GI immune response is the result of many complex interactions occurring within the gut to promote a tolerant approach to orally introduced antigens. Due to the many possible routes of entry and subsets of immune cells that may play a role in the immune response, mechanisms of gut immunology are just beginning to be understood. Within the large surface area and complexity of the GI tract, the host immune system must function to respond correctly to ingested antigens, without wasting resources on inflammatory responses to nonpathogenic bacteria or self-antigens. Oral tolerance is the host solution to the constant exposure to diverse antigens within the gut. One remarkable quality of the GI tract is that the host immune system does not mount an inflammatory response against the indigenous microbiota in the gut under normal circumstances. Further research is necessary to begin to understand how all of the players in the immune response work together to create the appropriate response to ingested antigens.

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CHAPTER 2

The Commensal Microbiology of the Gastrointestinal Tract

Janet M. Manson, Marcus Rauch and Michael S. Gilmore*

Abstract

The gastrointestinal (GI) tract is a dynamic environment and therefore the stability of the commensal community, or microbiota, is under constant challenge. Microscopic observations have revealed that the majority of bacteria present in the GI tract are not detected using standard culturing techniques, however with the application of culture-independent techniques it has been estimated that between 500 to 1000 bacterial species inhabit the human GI tract. Numerically predominant organisms in the microbiota belong to two eubacterial divisions, the *Cytophaga-Flavobacterium-Bacteroides* (CFB) and the *Firmicutes,* and fall into three main groups; *Clostridium* rRNA subcluster XIVa, *Clostridium* rRNA subcluster IV and *Bacteroides.* The prevalence and diversity of bacteria in different areas of the GI tract is influenced by the different conditions at these sites and thus the microbiota of the stomach and jejunum varies with that of the large intestine. Additionally, host genotype, age and diet have all been shown to affect microbial diversity in the GI tract. The distal intestine harbours the highest bacterial cell densities for any known ecosystem. Characterizing the species composition of the healthy microbiota may be a key step in identifying bacterial or associated physiological conditions that are present or absent in an unhealthy microbiota.

Introduction

The human gastrointestinal tract (GI tract) is a complex and dynamic ecosystem due to the interplay between peristaltic movement, food particles, host cells and defence molecules and a vast array of resident microbes and their secreted products. The microbial population, estimated to total about 10^{14} bacteria, is 10 to 20 times greater than the total number of eukaryotic cells in the human body. It has been estimated that between 500 to 1000 bacterial species inhabit the GI tract.[1,2] Despite being complex in the number of species that occur in the GI tract, only 11 of the known 55 bacterial divisions are represented.[3-5]

Members of the GI tract exist in an open ecosystem and are classified into two groups.[6,7] Autochthonous organisms are classified as entrenched residents, that is, indigenous species that occupy a given ecological niche. Allochthonous organisms are transitory and do not occupy a niche but rather pass through the gut. When examining the GI tract microbiota, both resident microbes and transient bacteria, such as those found in fermented food products, can be detected. Therefore it is important to examine the ecology over time as the irregular detection of species can provide information on their association with the GI tract.

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The microbial community, or microbiota, play an important physiological role in human health and collectively can be viewed as a functional organ. The collective genome of the gut flora, termed the microbiome, contains biochemical pathways, some being essential for life that humans have not evolved—biosynthesis of essential nutrients such as vitamins K and B12, biotransformation of conjugated bile acids and degradation of dietary oxalates. Due to the high number of bacterial species present in the human GI tract the coding capacity of this microbiome vastly exceeds the human genome.[1] The presence of the gut microflora also enables usable calories to be extracted from otherwise indigestible polysaccharides. The release of butyrate as an end-product of fermentation plays an important role in the metabolic welfare of colonocytes and has been implicated in providing protection against cancer and ulcerative colitis.[8-10] Also, due to their occupation of available habitats and adhesion sites the indigenous (autochthonous) microflora prevent potentially deleterious allochthonous organisms from taking up residence. This phenomenon is known as colonization resistance.

The human GI tract is an open system consisting of several compartments that differ in their physiological condition and microbial population (Fig. 1). Different factors constantly challenge the stability of the microbial community: (1) Rapid turnover of the intestinal epithelium and overlaying mucus; (2) Exposure to peristaltic activity, food molecules and gastric, pancreatic and biliary secretions; (3) Exposure to transient bacteria from the oral cavity and esophagus. In the stomach and bowel the prevalence and diversity of bacteria is influenced by several factors, including pH, peristalsis, redox potential, mucin secretion and nutrient availability. The stomach and the upper two thirds of the lower intestine contain low numbers of microorganisms (10^3 to 10^4 bacteria per ml) which is due to low pH, swift peristalsis, high bile concentrations and the production of α-defensins by the intestinal Paneth cells.[7,11] In the distal small intestine, the microflora begins to resemble more closely that of the colon. The distal intestine is largely anaerobic. It harbours the majority of the microorganisms in the gut and achieves the highest cell densities recorded for any ecosystem.[12]

Culture-Dependent Versus Culture-Independent Techniques

Early studies of the GI tract microbiota were based solely on culture-dependent techniques and microscopy. Most of these studies examined the composition of feces. Among the most common genera detected in these studies are *Bacteroides, Clostridium, Fusobacterium, Eubacterium, Bifidobacterium, Lactobacillus, Peptostreptococcus, Escherichi* and *Veillonella*.[7] The samples did not appear to be diverse in bacterial species as examined by these methods, with an average of only 20 to 40 bacterial species found.[13,14]

More recently, microscopic observations using the DNA stain DAPI (4', 6'-diaminido-2-phenylindole) revealed that 60 to 80% percent of the bacteria present in fecal sample were not enumerated using culture-based methods.[15,16] Comparing culturable bacteria versus bacteria observed microscopically in fecal samples, Suau et al[15] found 10.6×10^{11} cells per g (dry weight) present in DAPI stained samples examined microscopically as compared to 2.2×10^{11} CFU per g (21%) detectable on nonselective medium grown anaerobically. Many organisms may be obligate syntrophs that will not grow in isolation. Additionally, strict anaerobiosis of some of the GI tract microbiota may result in plating inefficiency during sampling. Finally, intercellular adherence may reduce the number of organisms giving rise to colonies.

More recently, the application of cultivation-independent techniques based on 16S rRNA sequence identification has provided tremendous insight into the diversity of microflora resident in the GI tract. The genes for the 16S rRNA (16S rDNA) have both conserved regions (which permits design of universal primers) and variable regions (allowing identification and differentiation of different species). Sequence analysis of amplified and separately cloned 16S rRNA amplicons allows identification of bacterial phylotypes or operational taxonomic units (OTUs). Because of debate over what precisely constitutes or defines a species, sequences sharing 98% identity in the 16S rRNA genes are often defined as phylotypes or OTUs. Sequence information from 16S rRNA clone libraries from both feces and from other regions of the GI tract have indicated that a significant

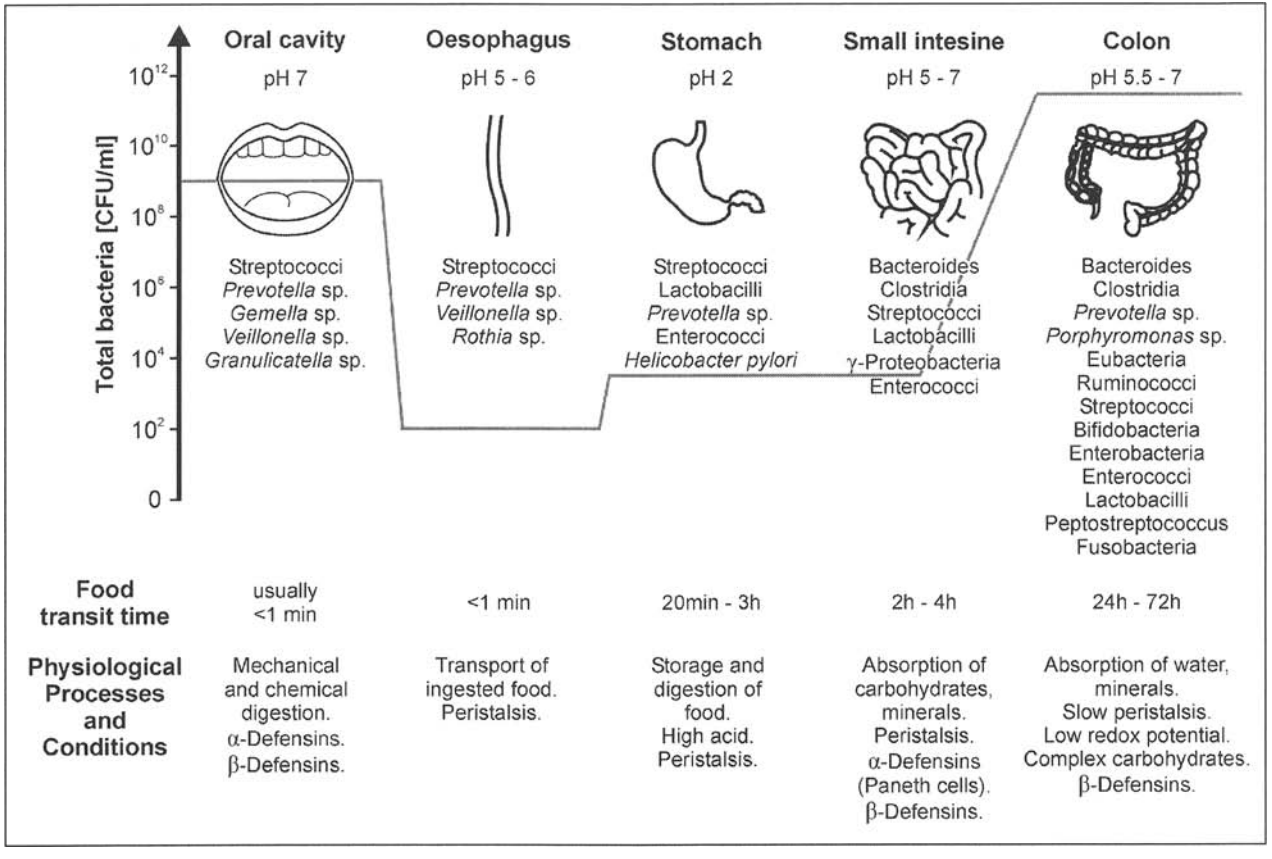

Figure 1. Compartments of the human intestinal tract: physiologic conditions and bacteria genera found therein.

fraction (60 to 80%) of bacteria have not been previously described.[15,16] Additional DNA-based techniques have been helpful in characterizing the human GI tract microbiota and its complexity, such as fluorescent in situ hybridization (FISH), denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) (see Table 1).

Bacterial Diversity

Major Groups

Of the 11 bacterial divisions detected in the gastrointestinal tract, eight divisions are rare.[3-5] Using 16S rRNA techniques, the numerically predominant organisms belong to two eubacterial divisions, the *Cytophaga-Flavobacterium-Bacteroides* (CFB) and the *Firmicutes*. A recent, comprehensive study by Eckburg et al[5] found that from 11,831 bacterial 16S rRNA sequences, 395 phylotypes were present. Of those, 93% were *Firmicutes* or CFB. Interestingly, of 1524 archaeal sequences from the same study, all belonged to one phylotype (*Methanobrevibacter smithii*).[5] This archaea is the numerically dominant methanogen isolated from humans that harbor a methanogenic flora.[17-19] In another study evidence was provided that the archea *Crenarchaeota* may also be found.[20]

The majority of the *Firmicutes* detected in the gastrointestinal tract fall into two main groups. The *Clostridium coccoides* group (also known as *Clostridium* rRNA subcluster XIVa[21]) consists of members of the *Clostridium, Eubacterium, Ruminococcus, Coprococcus, Dorea, Lachnospira, Roseburia* and *Butyrivibrio* genera. The *C. coccoides* group is generally predominant in the gut, consisting of 11 to 43% of total bacteria in several studies examining GI tract diversity.[22-29] A large number of species of this group are as-yet-uncultured bacteria. A recent study investigated the diversity of the *C. coccoides* group, using group-specific primers.[30] Of 972 clones analyzed, 139 OTUs were identified, of which only nine possessed sequences closely related to those of cultivated bacteria.[30] The *C. coccoides* group contains a large number of butyrate producers such as *Roseburia intestinalis*,[31] which are thought to contribute to intestinal health, as butyrate serves as the preferred energy source for colonocytes.[8,9] Bacteria belonging to this group were found to make up 80% of randomly isolated butyrate-producing anaerobes isolated from human feces.[32]

A second predominant group within the *Firmicutes* is the *Clostridium leptum* group. This group includes species belonging to *Clostridium, Eubacterium, Ruminococcus* and *Anaerofilum* genera. This group, also called *Clostridium* rRNA subcluster IV, is made up of highly oxygen-sensitive anaerobes and contains a high number of butyrate-producing fibrolytic bacteria.[21] A recent study by Lay et al[33] investigated the composition of the *C. leptum* subgroup using FISH and noted that 21% of fecal bacteria belonged to this group. Saunier et al[34] obtained similar results with *C. leptum* comprising 19% ± 7 of fecal bacteria. Of the *C. leptum* group, Lay et al found *Faecalibacteria prausnitzii* comprised 64%, followed by *Ruminococcus bromii* (12%), *R. flavecaiens* (1.8%) and *R. callidus* (1.4%).[33] Despite its initial designation, *Fa. prausnitzii* (formerly *Fusobacterium prausnitzii*) is not closely related to *Fusobacterium* species, having a G + C content ranging from 47 to 57%.[35] Oligonucleotide probing suggests that *Fa. prausnitzii*-related strains are among the most abundant in human feces.[36] Using culture-dependent techniques,[13] others have also found *Fa. prausnitzii* to be common among the human fecal flora with this phylotype sequence comprising 3.8 to 10% of clones in 16S rRNA libraries.[15,37] In the most comprehensive study thus far reported, the *Fa. prausnitzii* phylotype occurred in 1,556 of 11,831 16S rRNA sequences.[5] This was observed to be geographically independent, in five different countries *Fa. prausnitzii* was identified as the most abundant member of the *C. leptum* group, making up 13 to 17.6% of total bacteria.[33] Collectively, these results indicate that this phylotype may make an important contribution to GI tract ecology, especially since *Fa. prausnitzii*-like bacteria are capable of producing >10 mM of butyrate during fermentative growth.[38]

The third group most frequently identified among flora of the gastrointestinal tract is the *Cytophaga-Flavobacter-Bacteroides* (CFB) division. The *Bacteroides*, along with *Prevotella* and *Porphyromonas*, form a major branch of this phyla. *Bacteroides* are Gram-negative obligate anaerobes, with G + C compositions of 40 to 48%. Of the *Bacteroides* sequences identified by

Table 1. Description of commonly used culture-independent techniques for measuring bacterial diversity and distribution

16S rRNA Clone Libraries	TGGE/DGGE[a]	FISH[b]	Dot Blot
DNA is extracted from the sample, and universal primers are used to amplify 16S rDNA. The resulting PCR products are cloned into E. coli and sequenced. Advantages: Identifies previously unknown organisms and allows phylogenetic identification. Disadvantages: Biases in DNA extraction, PCR and cloning.	DNA is extracted from the sample. Primers (one containing a GC-clamp to stop complete denaturation) are used to amplify the 16S rDNA, and the PCR product is run on a denaturing or temperature gradient gel electrophoresis system. The G+C content of the 16S rDNA sequence determines how much denaturation occurs and affects how far the band runs on the gel. Bands of interest can be excised, cloned and sequenced. Advantages: Allows diversity comparison between many samples over time. Disadvantages: Biases in DNA extraction, PCR and cloning.	Bacterial cells in a sample are permeabilized to allow entry of a fluorescently labelled oligonucleotide probe. Numbers of target cells can then be counted, and the proportion of target cells can be determined by comparison with the total number of bacteria (DAPI stained). Advantages: Detection and enumeration of different bacterial populations. Disadvantages: Requires known 16S rRNA sequences.	RNA is isolated from the sample and blotted onto a membrane. The membrane is probed with a species-specific, radioactively labelled oligonucleotide and compared with membranes hybridized with a eubacterial probe (detecting all bacteria). The relative abundance of each bacterial species can then be determined. Advantages: Allows detemination of relative abundance of 16S rRNA. Disadvantages: Requires known 16S rRNA sequences.

[a]Temperature/Denaturing gradient gel electrophoresis. [b]Fluorescent in situ hybridization.

Eckburg et al.[5] *B. vulgatus* comprised 31%, *B. thetaiotaimicron* 12% and *B. distasonis* 0.8% and these, along with *B. fragilis*, are the most common species. Using PCR to examine the predominant culturable bacteria in feces, Matsuki et al[39] found that a majority of isolates belonged to the *B. fragilis* group (117/300). This high proportion may reflect the relatively high oxygen tolerance and ease of cultivation of the *B. fragilis* group. *Prevotella* is often associated with the oral cavity and

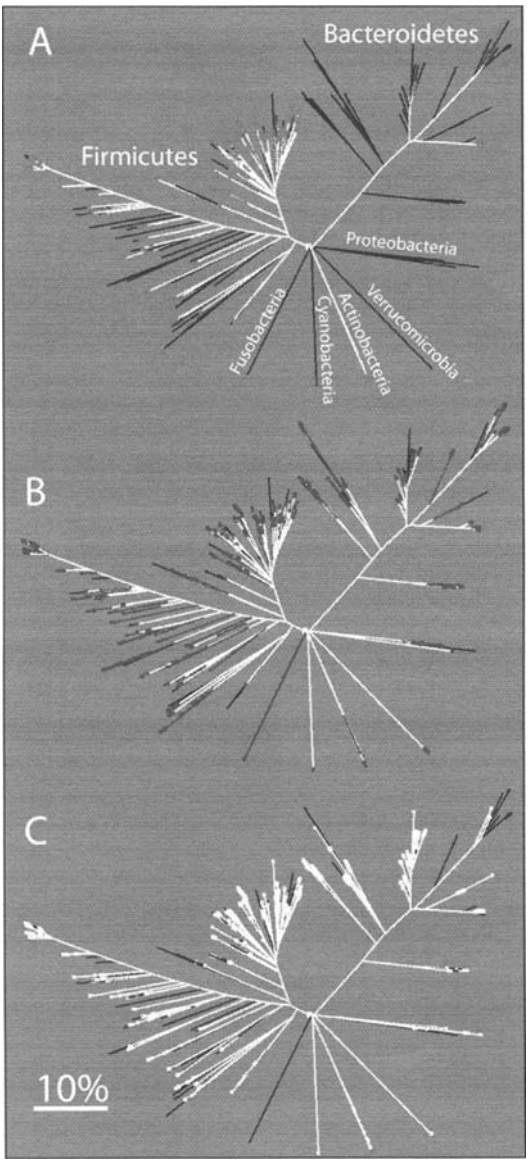

Figure 2. Variation in bacterial diversity within the colonic microbiotas of three healthy humans. Reprinted from Cell, Vol 126, Ley RE, Peterson DA, Gordon JI. Ecological and evolutionary forces shaping microbial diversity in the human intestine. 837-848, 2006, with permission from Elsevier.

its presence in the GI tract appears variable. Matsuki et al[40] found *Prevotella* in 46% of volunteers (21/46). However, those that were colonized contained high numbers ($\log_{10}$ 9.7 ± 0.8). Eckburg et al[5] found that the distribution of the *Bacteroides* branch of the CFB subgroup showed the greatest variability from host to host, with 99.8% of all *Prevotella* sequences isolated from subject B and 75% of *B. vulgatus* associated with subject A (Fig. 2).

Irrespective of culture independent method used, studies on GI tract ecology are in good agreement that the *Clostridium coccoides*, the *Clostridium leptum* and the *CFB* subgroups dominate among human fecal flora and the distribution of these three bacterial groups is summarized in Table 2.[15,22-28,33,37,40-44]

Minor Members

Using culture-based methods, *Bifidobacterium* was originally estimated to make up about 10% of the microbiota, however, due to the introduction of culture-independent methods, this number is now estimated to be much lower. Bifidobacteria are gram-positive rods, first isolated in 1899 from the feces of breast-fed infants.[45] Their presence has since been associated with a healthy microbiota and they are included in many food preparations with associated health-related claims. Langendjik et al[46] examined the prevalence of *Bifidobacterium* in human feces and upon comparison with the DAPI total cell counts, *Bifidobacterium* was found to account for 0.8% ± 0.4 of the total population. Approximately the same number of bifidobacteria were noted using both culture-dependent and independent techniques, suggesting that most fecal bifidobacteria in feces were culturable.[46] Based on these newer findings it was suggested that bifidobacteria abundance had been overestimated by 10-fold, a conclusion also drawn by others.[47] Using quantitative PCR, Matsuki found the average total bifidobacteria per gram of feces was $\log_{10}$ 9.4 ± 0.7 and the most common *Bifidobacterium* species isolated were *B. adolescentis, B. catenalatum* and *B. longum*.[48]

Table 2. Distribution of the three predominant bacterial groups in human feces, determined using different molecular techniques

Method	*C. coccoides*[a]	*C. leptum*[b]	*Bacteroides*	Reference
rRNA library	23.7-58.8%	11.0-22.7%	5.0-16.3%	Hayashi et al[37]
rRNA library	44%	20%	30%	Suau et al[15]
rRNA library[c]	43.3-48.7%	10.8-17.9%	20.5-35.1%	Hold et al[42]
Dot Blot	14% ± 6%	16% ± 7%	37% ± 16%	Sghir et al[23]
Dot Blot	22.8% ± 2.2%	13.0% ± 0.78%	8.0% ± 0.32%	Marteau et al[24]
TRAC[d]	42-43%	9-12%	NT[e]	Maukonen et al[29]
FISH	16.9%	NT	NT	Zoetendal et al[22]
FISH	29%	NT	20%[f]	Franks et al[28]
FISH/flow cytometry	28% ± 11.3%	25.2% ± 7.6%	8.5% ± 7.1	Lay et al[43]
FISH/flow cytometry	22.9% ± 9.9%	21.7% ± 7.7%	7.2% ± 6.2%	Lay et al[33]
FISH/flow cytometry	12.7-29.7%	NT	3.2-16.8%	Mueller et al[44]
RT-PCR	29%± 12%	15% ± 10%	11% ± 7.8%[g]	Matsuki et al[40]

[a]Refers to *C. coccoides* group (*Clostridium* rRNA subcluster XIVa). [b]Refers to *C. leptum* group (*Clostridum* rRNA subcluster IV). [c]Results obtained from human colonic tissue samples. [d]Transcript analysis with the aid of affinity capture. A technique involving the quantitation of bacterial 16S rRNA by hybridization with oligonucleotide probes, followed by affinity capture.[86] [e]Not tested. [f]Species-specific probes were used and this number represents only *B. fragilis* and *B. distasonis*. [g]Species-specific primers were used and this number represents only *B. fragilis*

A second minor bacterial group populating the GI tract are the lactic acid bacteria (LAB). As the name suggests, this group of bacteria produce lactic acid as an end product of fermentation. Typical LAB such as *Lactobacillus, Lactococcus, Enterococcus, Streptococcus* and *Leuconostoc* belong to the low G + C phylum of Gram-positive bacteria. The *Lactobacillus*-enterococci group of bacteria had previously been estimated—using culture-dependent techniques—to comprise approximately 2% of the bacteria in adult feces. Harmsen et al[27] performing FISH with a *Lactobacillus*-enterococci probe, placed this number closer to 0.01% of the total microbiota, indicating that earlier estimates were inflated because of the cultivability of these organisms. Other minor divisions of bacteria found in the GI tract include *Proteobacteria, Verrucomicrobia, Fusobacteria, Actinobacteria, Cyanobacteria, Spirochaetes, Deinococcus/Thermus, Deferribacteres* and *VadinBE97.*

Regional Colonization of the GI Tract

Stomach Microflora

The GI tract, from oral cavity to distal colon, represents a variety of habitats with the stomach being one of the most extreme. Older studies attempted to cultivate organisms from the gastric juices or mucosal biopsies. It was generally assumed that very few bacteria were able to survive in the strongly acidic environment of the stomach.[7,49] More recently culture independent studies of the stomach have been conducted to detect and quantify specific pathogens, such as *Helicobacter pylori,* which is an important member of the gastric biota due to its association with gastritis and peptic ulcer disease. In addition to DNA from *H. pylori,* TGGE analysis of gastric biopsy specimens has identified DNA from *Enterococcus, Streptococcus, Staphylococcus* and *Stomatococcus* which are all normal inhabitants of the respiratory tract and oral cavity.[50] Several TGGE bands, however, represented *Pseudomonas* species,[50] which are not usually associated with this environment, and *Pseudomonas* species have also been cultured from gastric aspirates from individuals suffering from gastric reflux.[51] A recent study by Bik et al,[4] however, failed to identify any Pseudomonas species and thus the association of this organism with the stomach is unclear. Bik et al[4] applied culture independent methods to identify 128 different bacterial phylotypes in the stomach. Analysis of 1833 clones from 23 adult subjects showed five major phyla are dominant in this environment: *Proteobacteria, Firmicutes, Bacteroidetes, Actinobacteria* and *Fusobacteria.* Among the 128 phylotypes, 50% represented uncultivated bacteria. Of those, 67% had previously been described in the oral cavity, suggesting the possibility of colonization from oroesophageal sources.[4] Of interest was one sequence belonging to the *Deinococcus/Thermus* phylum as this is the first report of this phyla being associated with a human source. It was suggested that some of these bacteria might have adapted to live in this environmental habitat as (1) the bacterial sequences found in the stomach were not simply a random sampling of those from oroesophyhageal sources, (2) a number of rRNA sequences found in the stomach did not appear to be related to oroesopyhageal sources and (3) 10% of the phylotypes found were previously uncharacterized. However, whether a distinct stomach-associated microflora exists, needs to be further verified.

Mucosa, Small Intestine and Cecum

In healthy humans the intestinal epithelium is covered by a layer of mucus, composed mainly of mucin. Because of the mucosal inflammation that occurs in Crohn's Disease and ulcerative colitis, several studies have attempted to characterize the microflora of the GI tract mucosa in health and disease and to examine microbial variation over the length of the small and large intestine.[52-56]

Study of the mucosa-associated bacteria, using culture-dependent techniques, found that *Bacteroides* species predominated in both proximal colonic and rectal biopsy samples, comprising 66% and 68.5% of total bacterial counts, respectively.[57] Croucher et al[58] found 49% of isolates cultured from the mucosa were *Bacteroides* and *Fusobacterium* species. Also cultured were gram-positive rods, including *Bifidobacterium* (27%), anaerobic cocci (20%) and *Clostridia* (4%).

Using culture-independent techniques, Wang et al[54] compared the diversity of mucosal biopsies from four areas of the human intestinal tract. 16S rRNA libraries from the distal ileum, ascending colon and rectum revealed *Bacteroides* (27-49%) and *Clostridium* clusters XIVa (20-34%) and

IV (7-13%).[54] It was noted that bacteria in the jejunum are very different from those in the distal ileum, ascending colon and rectum. Organisms predominant in the jejunum included species closely related to the streptococci (68% of clones from the region belonging to this genus) and of these 86% shared ≥98% similarity to *Streptococcus mitis*.[54] A second study by Hayashi et al[59] substantiated these findings by showing again that the jejunum and lower intestine comprised very different microbial communities. Using contents of the lumen rather than mucosal biopsies, the jejunal and ileal microbiota was found to consist of simple microbial communities of *Streptococcus, Lactobacilli, Gammaproteobacteria, Enterococcus* and *Bacteroides*.[59] The *Clostridium* clusters XIVa and IV predominated in the cecum, but were not detected in samples from the upper gastrointestinal tract. From this data it appears that the jejunum microbiota is very different from that of either the stomach or the distal ileum, with aerobes or facultative anaerobes predominating. A difference in microbial diversity in the cecum was noted by Marteau et al[24] who found that strict anaerobic bacterial populations such as the *Bacteroides, C. leptum* and *C. coccoides* groups were significantly lower than in the colon, while facultative anaerobes encompassing the *Lactobacillus-Enterococcus* group and *Escherichia coli* species represented large proportions of the rRNA amplified from the cecal contents (50% of rRNA).

Zoetendal et al[52] examined the distribution of bacteria in the gut mucosa of the colon using DGGE. Consistent with the above studies that found similar representations of flora from various points in the lower bowel, this study found relatively little regional variation. However, the organisms identified in the colonic mucosa differed from fecal samples and were patient specific, observations supported by others.[55,56,60] Interestingly, identical DGGE profiles were noted in both washed and unwashed biopsy samples, suggesting that the bacteria may be strongly adherent to the mucosa.[60] Using FISH, Swidsinski et al[61] found six bacterial groups adherent to the mucosa: *Bacteroides, Enterobacteriaeceae-E. coli, Brachyspira, Fusobacterium, Eubacterium rectale-C. coccoides* and *Enterococcus faecalis*.

When examining the colonic mucosa for specific organisms, regional differences have been observed. Using DGGE profiling of the *Lactobacillus*-like communities within the colonic mucosa, variation with sampling site was observed in four individuals.[56] The same study, using *Bifidobacterium*-specific primers, found DGGE profiles were host specific but did not vary with sampling site.[56] This suggests that the *Lactobacillus*-like microbiota varies with position in the colon but other bacterial populations may not. Zoetendal et al[52] found that when looking at *Lactobacillus* group-specific composition, three of ten individuals had minor differences between biopsy samples and it was suggested that changing conditions such a nutrient availability and pH in the GI tract could influence the presence or absence of certain *Lactobacillus* species.

Influences on Microbiota

Host Specificity

Bacterial diversity in the gastrointestinal tract appears to be host specific. Using a DNA microarray designed to detect 40 bacterial species, Wang et al[62] found that out of 11 fecal samples from different individuals, no two samples gave the same profile. Similar results have been shown by others.[5,16,52,63] Interestingly, two separate studies have further suggested that host genetics rather than environment may play the most important role in the bacterial composition of the GI tract.[64,65] Zoetendal et al[64] found that the similarity of DGGE profiles of identical twins were higher than in unrelated individuals and, additionally, that DGGE profiles of marital partners showed low similarity. Corroborating these findings, it was found that there were significant differences in TTGE profile similarity of faecal samples when comparing identical to fraternal twins and fraternal twins to unrelated controls.[65]

Diet

Diet appears to play an important role in microbial diversity in the GI tract. Hayashi et al[66] found marked differences in the fecal microbiota when comparing Japanese vegetarians to those with omnivorous diets. Of 183 'vegetarian' clones examined, *Bacteroides* made up only 6% and *Fa. prausnitzii* was

not detected in the 16S rRNA clone library or by cultivation. Finegold et al[67] also failed to detect this species in the feces of subjects with a vegetarian diet. Consistent with this association, a comparative study of fecal microbiota among Europeans noted that the Swedish study group was found to have the highest numbers of *Fa. prausnitzii*, their diet was characterised by high consumption of fish and meat.[44] As *Fa. prausnitzii* is normally a major component of the GI tract microflora, these findings suggest that diet may have a significant impact on microbiota composition.

Age Related Changes

In addition to diet, age has a significant impact on the GI tract microbiota, and large changes in bacterial composition are seen shortly after birth. During birth, the sterile GI tracts of neonates are colonized with bacteria both from the mother and the environment. After birth, the babies are continuously inoculated with new microbes by suckling and other contact, and breast-fed infants are inoculated with bacteria from both the breast skin and milk, that can contain up to 10^9 microbes per liter.[68] Immediately after birth, *E. coli* and enterococci are the most commonly isolated organisms from the GI tract of infants and these species dominate the microbiota for the first few days. It has been suggested that the presence of these bacteria leads to a reduced environment compatible with colonization by obligate anaerobic genera like *Bacteroides*, *Bifidobacterium* and *Clostridium*.[69-71] Corynebacteria, lactobacilli, micrococci and propionibacteria are also frequently identified within a few days after birth.[70,71] After the initial colonization, the infant flora changes rapidly, the composition being profoundly influenced by the diet. Formula-fed babies quickly develop a more complex microbial consortium that resembles that of adults, and is codominated by bifidobacteria, *Bacteroides* and to a lesser extent clostridia.[70] In contrast, the microbiota of babies solely fed with human breast milk shows lower complexity and is dominated by bifidobacteria.[72,73] The composition of the intestinal microbiota of breast-fed infants changes during weening. With the onset of dietary supplementation it begins to approximate that of formula-fed babies in which bifidobacteria are no longer the dominant genus. Differences between breast-fed and formula-fed babies are lost by the second year when the fecal microbiota begins to resemble that of adults.[69,70]

Most studies of the adult GI tract microflora have reported a stable bacterial community unless perturbed by variations in diet, disease or antibiotic treatment. With age, however, there are changes in diet and host immune system activity and a higher incidence of gastrointestinal tract infections is found. A study by Mueller et al[44] examined the intestinal bacterial community in aged European populations and compared it to the bacterial community structure of younger adults from the same region. They observed higher proportions of enterobacteria in all elderly volunteers regardless of geography. Lower levels of *Bacteroides* were noted in elderly Italians. Others have noted higher numbers of enterobacteria and lower numbers of anaerobic bacteria in the elderly in Asia.[74] Interestingly, a study comparing fecal microbiota composition and frailty noted that elderly individuals with high frailty scores had a seven-fold increase in numbers of *Enterobacteriaceae*.[75]

Future Study of GI Tract Ecology

To date the majority of information on GI ecology has been based on the phylogeny of the gut microflora with little attention to its associated physiology. A problem that arises from some of the molecular techniques that have been used to characterise bacterial diversity is that although the bacterial DNA is present, it does not indicate whether the organism is dead, alive or metabolically active. Separating viable active, viable inactive and dead cells in fecal samples using flow cytometry with a live/dead bacterial stain, viable cells were found to comprise approximately one half of fecal matter, while dead cells accounted for one third.[76] Interestingly, butyrate-producing bacteria were found to predominate in the active population, while *Bacteroides*, *Ruminococcus* and *Eubacterium* were more abundant in the dead fractions.[76] Taking another approach to identify metabolically active bacterial populations, Tannock et al[77] used PCR-DGGE to compare bacterial community profiles generated with RNA (a surrogate measure of live bacteria) compared to DNA (a measure of total live and dead cells) as the amplification template and noted marked differences in profiles.

Although 16S rRNA gene surveys have helped to define microbial diversity in the gut, they provide little information about complex interactions between these microbes. A metagenomic approach has recently been employed to ascertain differences in fecal bacterial composition in people with Crohn's Disease (CD).[78] Analysis of the ribotypes from two fosmid libraries constructed from total DNA showed a global loss in diversity in CD patients and it was suggested that the loss of butyrate producers could upset interactions between epithelial cells and the microflora in CD patients.[78]

Information from genomic sequences of GI tract bacteria should begin to provide valuable insight into the physiology of organisms, especially those difficult to culture in vitro. Currently, however, genome sequence information is available for only a small number GI tract bacteria. Sequence from the bacteria *Lactobacillus plantarum* and *E. faecalis* has revealed a very large number of PTS-type transporters for the acquisition of exogenous sugars, and both these species predominate in the small intestine.[79,80] In contrast, genomes of bacteria that predominate in the large intestine contain a large content of genes involved in the utilization of complex carbohydrates. For example, the genome of *Bifidobacterium longum* has a large number of predicted proteins for catabolism of oligosaccharides.[81] *Bifidobacterium* is found in high numbers in breast-fed infants and has over 40 predicted glycosyl hydrolases whose predicted structures cover a wide range of di-, tri- and higher order oligosaccharides. Besides lactose, human milk contains over 80 diverse oligosaccharides and this may select for the presence of *Bifidobacterium*.[81] Another inhabitant of the lower GI tract is *Bacteroides thetaiotaimicron* and the genome has been found to contain 172 glycosyl hydrolases, 11 enzymes involved in degrading host-derived products, 163 outer membrane polysaccharide binding proteins as well as 20 specific transporters.[82] Additionally, this organism also contains 50 extracytoplasmic function (ECF)-type sigma factors and appears to have an extensive array of diverse mechanisms to adapt to shifting nutrient availability.[82] *Bacteroides fragilis* also contains a large number of genes involved in the acquisition of complex carbohydrates. This organism is more often associated with the GI tract mucosa and, interestingly, has numerous DNA inversion mechanisms to generate a wide range of cell surface structures.[83] Additionally, *B. fragilis* has been shown to use surface fucosylation to enhance colonisation, as L-fucose is an abundant surface molecule of intestinal epithelial cells.[84] Both of these mechanisms may aid the bacterium with immune evasion in its interactions with the intestinal mucosa.

To further expand our knowledge of the GI tract ecosystem, Gordon et al[85] and others have proposed a human gut microbiome initiative to deep draft the genome sequences of 100 GI tract associated bacteria. The acquisition of new information on the complex physiological interactions that take place in the GI tract could indicate various factors that may be involved in both health and in disease. Additionally defining the "healthy" microbiota could be a key step in identifying bacterial or physiological conditions that are present or absent in an "unhealthy" microbiota. The importance of the intestinal microbiota to human health has long been recognized and the advent of new technologies provides us with the tools necessary to begin to understand this vital "organ".

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CHAPTER 3

Overview of the Gastrointestinal Microbiota

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Abstract

The community of microbes that inhabits the mammalian intestinal tract exists in a symbiosis with their host. The structure of this community represents the combined effects of selection pressure on the part of the host and on the part of the microbes themselves. Through recent advances in the field of microbial ecology we are beginning to understand the forces that shape this complex community. We will review what is known about the interaction between the host and the indigenous microbial community. Following this discussion we will introduce methods that have been used to study the structure, function and dynamics of this community.

Introduction

The mammalian gut is inhabited by a complex community of microbes, collectively referred to as the microbiota.[1] Once thought of as a collection of freeloading "commensal" organisms that simply found a ready source of food, it is now appreciated that the relationship between the host and the microbiota is an intricate mutualistic symbiosis.[1-3] In return for secure environmental niches, the microbiota provide a number of key functions that contribute to the proper functioning of the host gastrointestinal tract.

In this chapter the concept of the microbiota as a part of a complex ecosystem comprised of the microbiota and the host epithelium and immune system will be presented. We provide a broad overview of the composition of the gut microbiota, including descriptions of a variety of culture-independent techniques that have been used to examine the structure and function of this community of microorganisms. Some of these strategies are illustrated with examples from our own work examining the microbiota of humans with antibiotic-associated diarrhea. We suggest that a detailed understanding of the structure/function relationships of the intestinal microbiota will have ramifications in rationale design and use of probiotics, since only with an understanding of the "natural balance" of the microbiota of the gastrointestinal tract can we effectively manipulate this ecosystem in a beneficial manner.

Structure of the Intestinal Microbial Community

It has been estimated that each person is inhabited by a microbiota consisting of 10^{14} organisms, outnumbering the number of host cells by an order of magnitude. The gastrointestinal (GI) tract is the home for the majority of these organisms.[1,4] Each segment of the GI tract appears to be populated with a distinct, stable community of microbes, with the highest density in the colon with an estimated density of 1×10^{12} organisms per gram (dry weight) of feces. For the remainder

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of this chapter, we will focus on the bacterial microbiota, although it should be noted that Eukaria (e.g., yeasts), Archaea and viruses are also members of this ecosystem.

One aspect of the indigenous GI microbiota that was apparent early on was that the community was quite diverse. For the discussion here, we will define diversity simply as "the variety and abundance of species in a defined unit of study".[5] Although species concepts for microbes are frequently incongruous with those of plants and animals, diversity measures can still be used effectively to describe and compare microbial communities. Diversity is composed of two key components, richness and evenness. Richness refers to what is likely the most intuitive aspect of diversity, namely the total number of species in the unit of study. Evenness on the other hand describes relative differences in the abundance of various species in the community. We will return to these concepts when we introduce the measurement of ecologic variables to describes different microbial communities.

Early measurements of the diversity of the GI microbiota came from culture-based studies that used the techniques developed by Hungate to cultivate strictly anaerobic microbes. It is from such studies that the often-quoted number of 400-500 distinct species in the gut microbiota was derived.[6] It is also from these early culture-based studies that it was estimated that the majority (up to 90%) of the different bacterial species present could be cultivated. More recent culture-independent studies of the intestinal microbiota suggest that these early studies largely underestimated the total species richness and overestimated the ability of current culture methods to cultivate the majority of organisms present.[4]

Data from culture-based and culture-independent analyses also suggest that there is significant individual-to-individual variation in the diversity of the gut microbiota, although the community within an individual appears to be relatively constant over time.[4,6-9] Although this individual variation suggests that the study of such large, intrinsically and extrinsically diverse communities presents intractable problems, recent work also suggests that at higher levels (i.e., ecosystem) of organization, principles are operating that allow one to study structural and function aspects of the entire community.[1,10,11]

For example, when using analysis of 16S rRNA-encoding gene sequence data to examine similarities between bacterial communities, one common definition of an operational taxonomic unit (OTU—roughly, the sequence-based equivalent of the taxonomic classification "species") is a group of organisms that share >97% sequence identity.[12] With this definition of OTU set at 0.97 sequence identity, a comparison of almost any two microbial communities obtained from individuals, or even from different anatomic sites within one individual, indicates that the communities are distinct. However, if the analysis is repeated and instead the communities are compared at the level of bacterial phyla, most normal individuals have quite similar communities with the majority of organisms belong to two phyla, the Firmicutes and Bacteroidetes. In fact, among the roughly 80 bacterial divisions described based on 16S rRNA-encoding gene analysis, only eight have ever been identified within the gut community. Perhaps even more striking is the fact that this applies not only to the human gut, but similar deep taxonomic structure is also encountered in the mouse intestine, implying that extreme selection pressure is shaping the structure of the microbial community in most (if not all) mammalian intestinal tracts.[10]

Functional Aspects of the Intestinal Microbiota

What is the nature of the selection pressure on the community structure of the gut microbial community? Clues to the forces that shape the community can be found by examining some of the proposed roles that the microbiota play in the intestinal ecosystem and the specific interactions they have with the host. One obvious force that has direct correlation with macroecologic systems is that stable ecosystems are thought to have well-organized and stable trophic structures.[13] The flow of nutrients between different members of a given ecosystem is influenced by and can in turn influence the members of the ecosystem. In the gut, it turns out that not only do the microbiota have access to a ready supply of nutrients (hence their common designation as "commensals") but metabolic products produced by the microbiota are in turn utilized by the host. This indicates

that for many members of the gut microbiota, their relationship with the host might best be considered to be mutualistic.[11] For example, members of the Firmicutes ferment nondigestible starch into short chain fatty acids, in particular, butyrate. Butyrate in turn is the preferred energy source for colonic enterocytes and also is thought to contribute to gut homeostasis through modulation of cell function.[14-18]

Through an incompletely understood set of signals, the microbiota also contribute to the normal development of the gut. Mainly through studies on gnotobiotic mice, the role of the microbiota in driving aspects of postnatal gut maturation has been delineated. During the time of weaning, there is a shift in the intestinal glycoconjugate repertoire, from glycans that terminate with sialic acid to those that terminate with the sugar fucose. Interestingly, this shift does not occur in germ free mice, but it can be induced by the monoassociation of these mice with the bacterium *Bacteroides thetaiotaomicron*.[19,20] It is noteworthy that the genome of *B. thetaiotaomicron* codes for an impressive array of mechanisms for the acquisition and utilization of dietary polysaccharides and that gene expression analysis reveals that the organism can shift its metabolism to utilize host mucus glycans when polysaccharides are absent.[21-22] In response to colonization with *B. thetaiotaomicron,* germfree mice modulate the expression of host genes important in nutrient formation, mucosal barrier function and angiogenesis.[23]

Although trophic interactions within the gut are readily obvious and have the most direct correlation with macroecologic systems, another important interaction between the microbiota and the host involves the host immune system. As will be discussed in later chapters, it is clear that the microbiota have the ability to stimulate beneficial as well as deleterious host immune responses. Less is known about how the host immune system can shape the community structure of the indigenous microbiota. One recent study, using the culture-independent T-RFLP method (see below) to "fingerprint" the microbiota of the terminal ileum showed that the lack of secretory antibodies in adult $pIgR^{-/-}$ mice did not alter the composition of the microbiota compared to wild type animals.[24] Conversely, another group using 16S clone library analysis showed that mice that lacked hypermutated IgA (due to a defect in activation-induced cytidine deaminase) had altered intestinal microbiota, most notably an expansion of the segmented filamentous bacteria.[25]

The influence of host genotype on the structure of the microbiota isn't restricted to immunologic function. It was recently demonstrated that mice deficient in the leptin gene had altered diversity of their microbiota manifested as inversion of the relative abundance of Bacteroidetes to Fimicutes compared to wild type littermates.[10] Interestingly this study also showed that while evenness was influenced by genotype, species composition reflected a maternal influence, demonstrating that the microbiota can be inherited vertically (particularly for mice raised in barrier facilities with sterilized food and water).

The effect of other host factors has been examined. In one study quantitative culture demonstrated that the status of the maternal adaptive immune system did influence the intestinal microbiota of suckling mice.[26] Similarly, a study that used hybridization probes targeting *Bifidobacterium* and *Lactobacillus* species showed that the administration of probiotics and breastfeeding in infants had an effect on the composition of the fecal microbiota.[27]

Methods to Study the Structure and Function of the Gut Microbiota

Limitations imposed by culture-based surveys of the gut microbiota gut have been circumvented by the application of molecular methods based on the direct extraction and analysis of nucleic acids from the microbiota (Fig. 1). The first step in analyzing the structure of such communities is frequently a survey of PCR-amplified 16S ribosomal RNA (rRNA) genes. This is a particularly useful gene to assess the composition of a microbial community due to the presence of conserved regions in the gene that are conserved amongst microbes and serve as convenient targets for amplification primers, coupled to the availability of a large data set of rRNA sequences that are available for comparison; there are currently more than 250,000 aligned, bacterial rRNA gene sequences in the Ribosomal Database Project.[28]

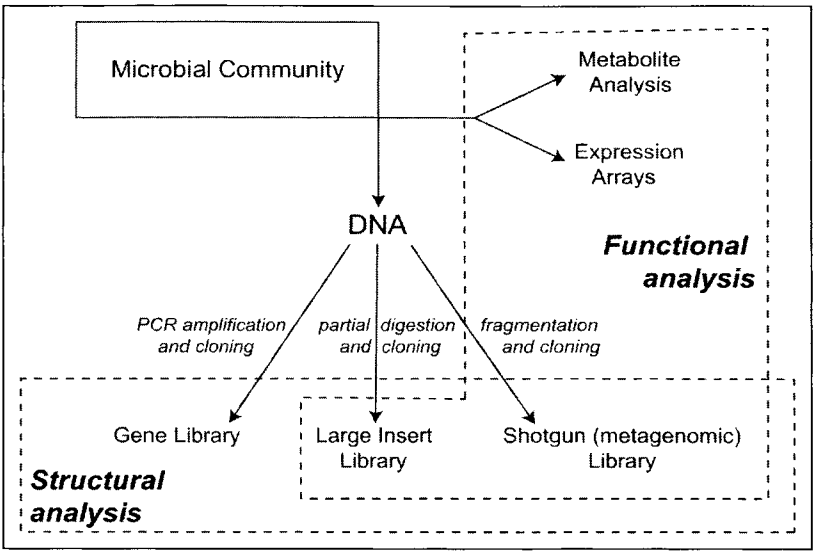

Figure 1. Molecular approaches for interrogating the structure and function of microbial communities. DNA-based approaches provide a cultivation independent assessment of community structure and metabolic potential, while RNA- or protein-based methods offer the opportunity to document expression of that potential under selected environmental condition.

While sequencing of rRNA genes provides the greatest resolution for phylogenetic identification of the resident microbes, high-throughput community fingerprinting approaches such as T-RFLP provide an overview of community structure that permits the simultaneous analysis of dozens of samples of the microbiota. In T-RFLP, one of the amplification primers is labeled with a fluorochrome; then following amplification, the pool of amplicons is digested with a restriction enzyme. The resulting mix of DNA fragments is separated based on size using a DNA sequencer, with only the fluor-containing terminal fragments subsequently detected due to their fluorescence. The resulting chromatogram (Fig. 2) reveals terminal restriction fragments (TRFs) from the more abundant members of the community. Upon inspection of a T-RFLP chromatogram, it is readily apparent that the method captures the ecologic diversity of the community. The number of TRFs is an indication of the number of different OTUs present in the community (i.e., richness) while the relative peak heights provide and indication of relative abundance (i.e., evenness).

While detailed methods for the construction and analyses of clone libraries and T-RFLP fingerprints of the colonic microbiota are readily available and these approaches are frequently used, [29-33] concerns about the interpretation of the results from these methods have been raised.There is documented potential for bias during PCR amplification as well as strategies, including decreased cycle number, to minimize bias.[34-36] However due to the idiosyncratic nature of molecular surveys that employ different amplification primers and DNA of different purity that is extracted from communities with varying degrees of complexity, there is unlikely to be a single, bias free procedure that is broadly application. As with any survey method, replication is an essential component of reliable nucleic acid based measures of community structure.

With advances in DNA sequencing technology, it is now feasible to extend beyond single gene surveys and query the entire genetic diversity present in the microbiome through the construction of large insert clone libraries or shotgun libraries.[37-38] These approaches do not include amplification before the construction of clone libraries and so avoid the potential bias of this initial step. More importantly, the libraries include information not only about the phylogenetic composition of a microbial community, but reveal the metabolic potential of the community as well. For instance,

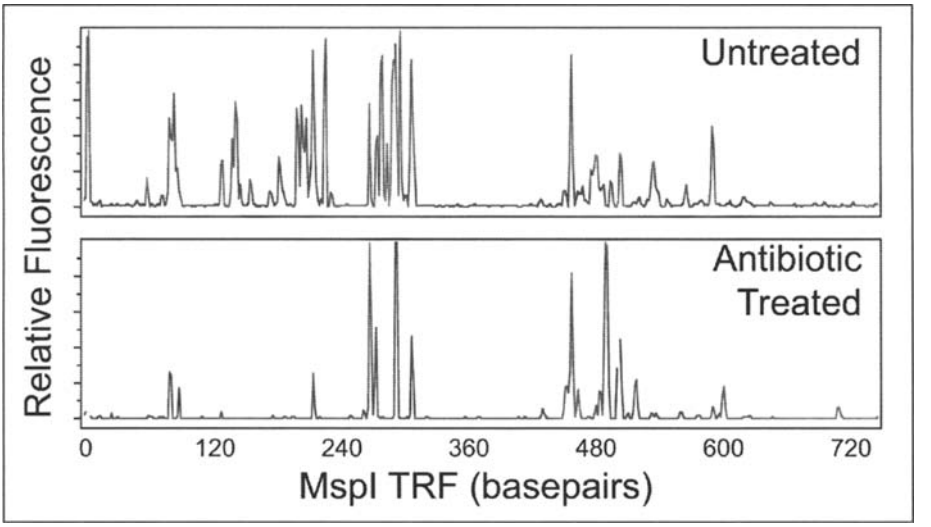

Figure 2. T-RFLP traces demonstrating the ability to provide a community fingerprint of the mucosa-associated microbiota from the cecum of a mouse. Compared to the T-RFLP profile from a control mouse (top), the T-RFLP profile from a mouse treated with antibiotics (metronidazole, amoxicillin and bismuth) has decreased diversity, most notably due to the decrease in the total number of terminal restriction fragments (peaks).

the genes involved in the formation of butyrate, an abundant short chain fatty acid in the gut, were enriched in clone libraries constructed from DNA extracted from the microbiome of the human GI tract.[38] This finding is consistent with the proposed role of the micobiome in providing colonocytes with this favored carbon and energy source.

While knowing the composition and metabolic potential of the microbiome can be useful in determining factors that influence the diversity, linking the structure of microbial communities with its function has the potential to exert the most profound influence on our understanding and successful manipulation of the microbiota. Again, direct extraction of nucleic acids, this time with a focus on mRNA, provides a window to view the fraction of the metabolic potential that is being expressed at a particular time and location in the GI tract. A recent application of whole-genome transcriptional profiling combined with mass spectrometry revealed that the presence of a methanogenic archaeon altered gene expression of a gut bacterium and thus has the potential to influence the host's energy harvest from dietary glycans.[39] Such functional analyses, coupled with structural analyses of the gut microbiota enhance our capacity to understand the role of the gastrointestinal microbiota in health and disease.

The Microbiota in the Context of the Intestinal Ecosystem

The discussion to this point has advanced the concept that the microbiota of the intestinal tract is not merely a random collection of "commensal" organisms that take advantage of a readily supply of nutrients. Instead, the indigenous gut microbiota are part of an intricate ecosystem comprised of the indigenous microbiota, the host mucosal epithelium and elements of the host immune system. As a stable ecosystem, there are interdependencies between the various components that contribute to the survival of each individual element. As a corollary to this idea, each component has evolved in a manner to survive within this ecosystem. Therefore, study of one component can provide insight into the function of the entire ecosystem. To illustrate these concepts, we will provide examples from our laboratories examining changes in the gut microbiota in the setting of antibiotic-associated diarrhea (AAD).[32]

Many patients who are treated with antibiotics subsequently develop diarrhea. A proportion of patients with AAD (estimated at about 25%) develop disease secondary to the presence of the toxin-producing bacterium *Clostridium difficile*. Both patients with *C. difficile*-associated diarrhea (CDAD) and nonCDAD are thought to develop disease secondary to antibiotic-mediated alteration of the gut microbiota. It is this hypothesis that has prompted clinical trials of probiotics in both CDAD and nonCDAD. A recent meta-analysis of these trials concluded that probiotics can be used to treat CDAD and prevent nonCDAD.[40]

To investigate if study of the fecal microbiota could provide insight into the pathogenesis of nonCDAD, we examined the microbial ecology of fecal specimens from a patient who developed diarrhea while taking amoxicillin/clavulanic acid.[32] Clone libraries of 16S rRNA-encoding genes were constructed from fecal DNA harvested from the first voided stool after antibiotics (which represents the baseline community), four days after initiation of therapy and 28 days after initiation (two weeks after the end of the 10-day course of antibiotics).

A total of 239 sequences were used in the final analysis, 84 from Day 0, 72 from Day 4 and 83 from Day 28. Given the relatively small number of sequences from three separate "communities" (i.e., the three sampling times), we could present the data in the form of a phylogenetic tree (Fig. 3). Examination of this tree shows that although this representation of the data can provide useful information, it also demonstrates that as the number of individual 16S continues to rise, the "tips" of the tree (i.e., individual clones) become increasingly crowded and difficult to discern. One solution to help reduce the complexity of the data is to group the data into phylogenetically coherent groups, as represented by the multiple bar graphs in the figure. In this case, the grouping of clones was based on observed clusters whose grouping was supported by bootstrap values. Bootstrap values provide nonparametric statistical analysis of the groupings that are encountered in a phylogenetic tree.[41] In brief, the data are randomly resampled multiple times, each time reconstructing a phylogeny. In essence, the bootstrap values indicate the percentage of resulting phylogenies that produce the same exact grouping seen in the original.

Although phylogenetic representations of 16S clone library surveys are commonly used and can provide important insight into the data, scientists who are not trained in the interpretation of phylogenies can find such representations to be problematic.[42] Additionally, as pointed out above, even with use of methods such as clustering and bootstrapping, it can be difficult to represent extremely large datasets. With the advances in DNA sequencing technology, extremely large datasets of 16S rRNA-encoding gene sequences are being assembled. Even when phylogenies are constructed using only "unique" phylotypes (defined based on a set percentage sequence similarity), when a large number of communities is being compared, visual examination of such representations can be daunting.[4]

As detailed above, the use of numerical methods to analyze ecological methods has been developed for the analysis of ecologic datasets, initially applied to macroecologic systems. One method that provides an alternative way to visualize the diversity present in a clone library is through the construction of rarefaction curves.[43] Rarefaction analysis is a method that allows comparisons between communities primarily based on richness. In brief, the rarefaction process

Figure 3, viewed on following page. Phylogeny showing the distribution of 16S rRNA-encoding gene sequences from clone libraries constructed from stool DNA samples obtained from a patient prior to antibiotic therapy (Day 0-red), during therapy (Day 4-green) and two weeks after discontinuation of therapy (Day 24-blue). Brackets outline major clusters of organisms and the adjacent bar graphs document the distribution of clones in each cluster at each time point. Named species are representative type species downloaded from the Ribosomal Database Project and inserted into the tree to provide taxonomic reference points. These reference species do not contribute to the number of clones depicted in the bar graphs. The scale bar represents evolutionary distance (10 substitutions per 100 nucleotides). The tree was constructed using neighbor-joining analysis of a distance matrix obtained from a multiple-sequence alignment performed using the ARB suite of programs. Bootstrap values were calculated using the MEGA2 program.

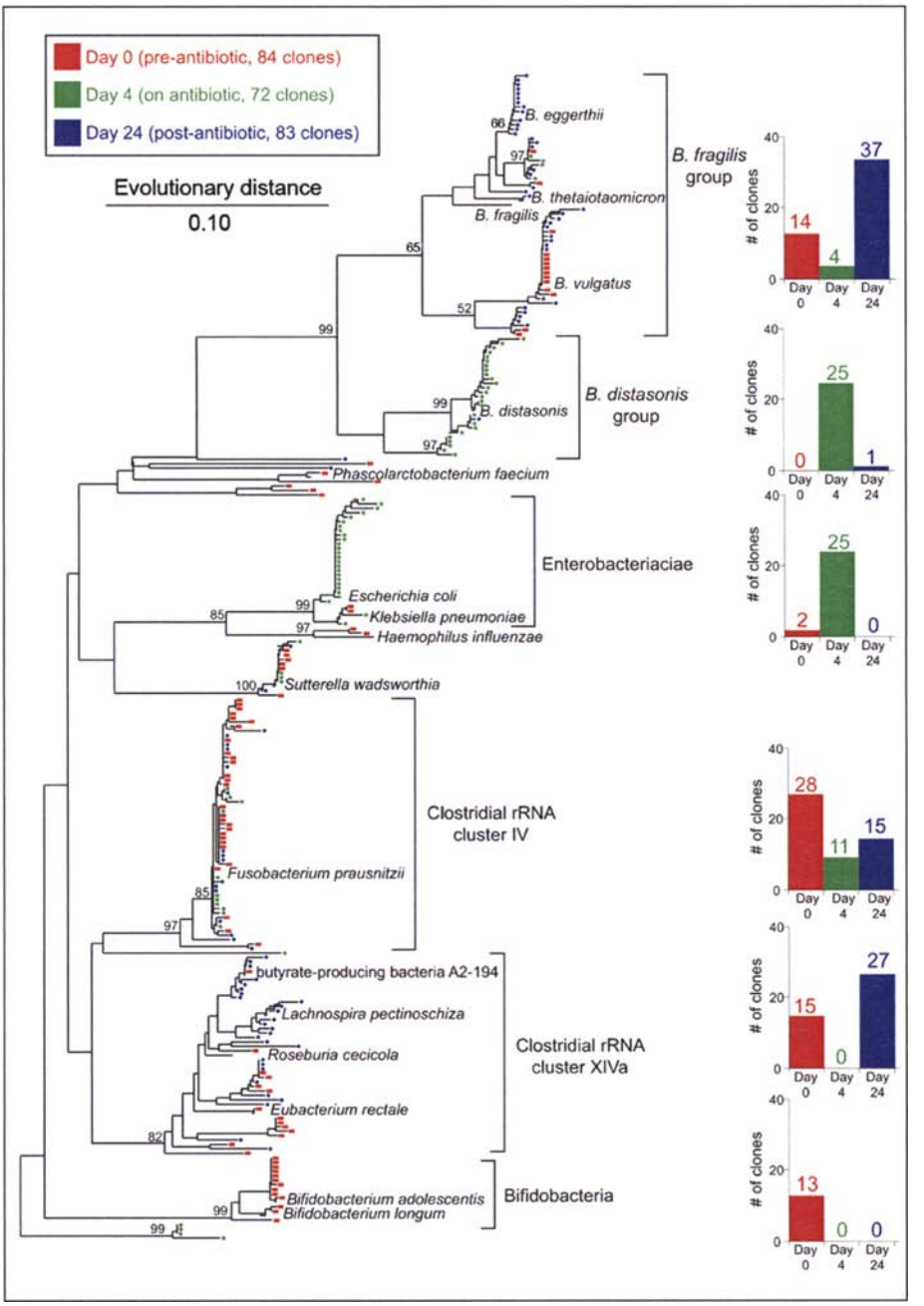

Figure 3, legend viewed on previous page.

involves iterative resampling of a given population dataset consisting of N members. Sampling is done without replacement and this will generate an estimate (along with confidence intervals) of the expected number of OTUs encountered in a subset n of the entire population represented

by N individuals. The curve is constructed by plotting the average number of OTUs represented by 1, 2, ...N individuals. A strength of rarefaction analysis is that it allows comparison between libraries that have been sampled with differing intensity.[44]

We constructed rarefaction curves for each 16S clone library from the antibiotic-associated diarrhea patient using the program DOTUR.[12] Examination of the curves (Fig. 4) reveals that antibiotic administration resulted in a decrease in the overall richness of the community, as the rarefaction curve from the Day 4 library lies below the Day 0 library. Once antibiotics were stopped, the rarefaction curve returned the original, implying that species richness was restored once the community was given a chance to recover. This conclusion is supported by the calculation of the estimated species richness for each library based on the method of Chao.[45] As discussed above, this method provides an estimate of the lower-bound of the actual richness in a partially sampled community. Using an OTU definition based on 3% sequence divergence employing the DOTUR program, the Chao1 estimate of richness is 67 for the Day 0 library, 25 for the Day 4 library and 59 for the Day 28 library.

Although comparisons of species richness can be useful, it is often informative to compare communities based on richness and evenness. Furthermore, comparison between communities based on rarefaction does not take into account as to whether specific OTUs are present in the different communities. It is possible that two communities can have the same overall structure and thus will

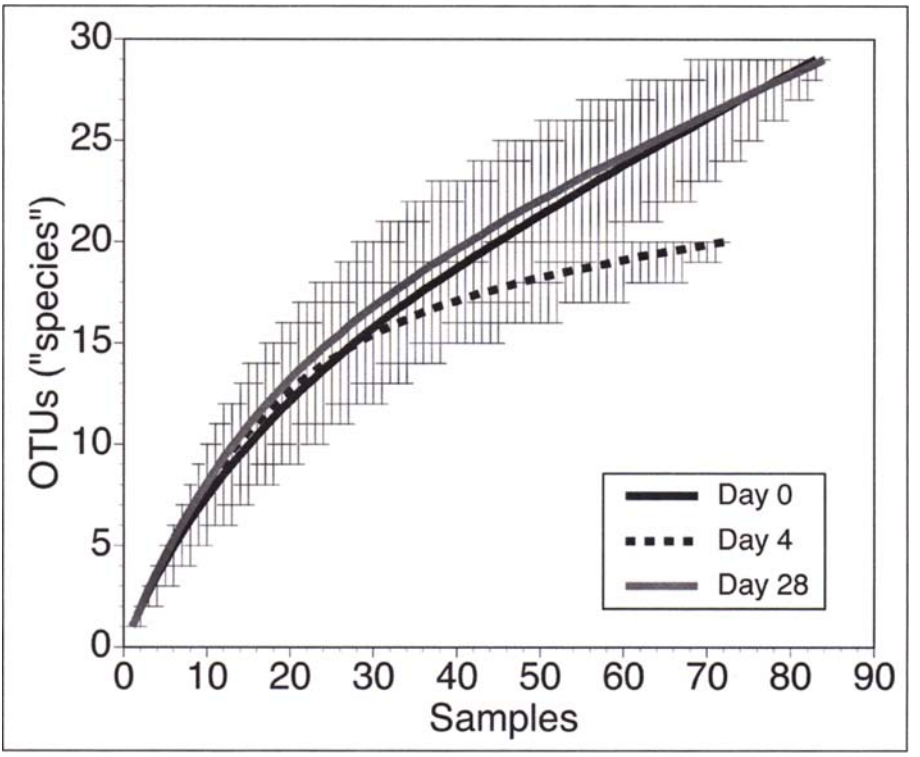

Figure 4. Rarefaction analysis comparing OTU richness in the three 16S libraries constructed from the patient with antibiotic-associated diarrhea. Antibiotic administration was associated with a decrease in overall species richness and this resolved following the discontinuation of the antibiotic treatment. The curves represent the average number of OTUs encountered during iterative resampling of the original clone data with 95% confidence intervals depicted by the error bars.

have identical rarefaction curves, but there are no OTUs that are present in both communities. In the example we are discussing here, there are shared OTUs in all three libraries, but even in case of the Day 0 and the Day 28 library, there are differences that are not captured by rarefaction analysis. *Bifidobacteria* were identified in the Day 0 library, representing approximately 16% of the clones but were not encountered in the Day 28 library despite having overlapping rarefaction curves.

In order to compare populations not only based on richness, but also in terms of evenness and the presence of shared OTUs we can use one of a number of beta-diversity indices. An example of such a metric is the Bray-Curtis distance measure.[46] Using such a distance metric, all pair-wise comparisons can be made between a set of communities and the results can then be displayed in a tabular format or in the form of a dendrogram. The latter representation of data can be useful in that it can provide visual evidence of clustering or grouping. When Bray-Curtis distances for the three clone libraries from the patient with AAD are depicted in dendrograms format, it is once again clear that antibiotic administration significantly changes the community structure of the fecal microbiota and that once antibiotics are discontinued, there is a return towards the baseline status (Fig. 5). However, as opposed to only looking at overall species richness, as was done with rarefaction analysis, this analysis shows that discontinuation of antibiotics resulted in a community structure that was more similar to the baseline community, but still distinct. Again, examination of the phylogeny constructed in Figure 1 supports this conclusion. For example, in addition to the lack of reappearance of *Bifidobacteria*, in the Day 28 library there was a proportionately greater amount of *B. fragilis* and Clostridial group XIVa organisms and an under representation of Clostridial group IV organisms compared to Day 0.

In Figure 5, two dendrograms are shown, demonstrating the effect of changing OTU definitions on analysis. In the first analysis, all sequences that shared ≥97% sequence identity were considered to belong to a given OTU. In the second analysis, the OTU cutoff was decreased to ≥80% sequence identity. As stated before, this roughly corresponds to the conventional "species" and "family" taxonomic divisions. Although changing OTU definitions will alter the calculated Bray-Curtis distance, the relationship between the communities remains similar in that the Day 0 and Day 28 communities are most similar and divergent from the Day 4 community. Although when considering rRNA-encoding gene sequence alone, OTU definition may appear to be somewhat arbitrary, it can become important when considering ecosystem function. It has been noted that although there appears to be significant diversity among the gut microbiota at the level of species and strain, there is relatively little divergence at deep phylogenetic divisions.[4,10] It has

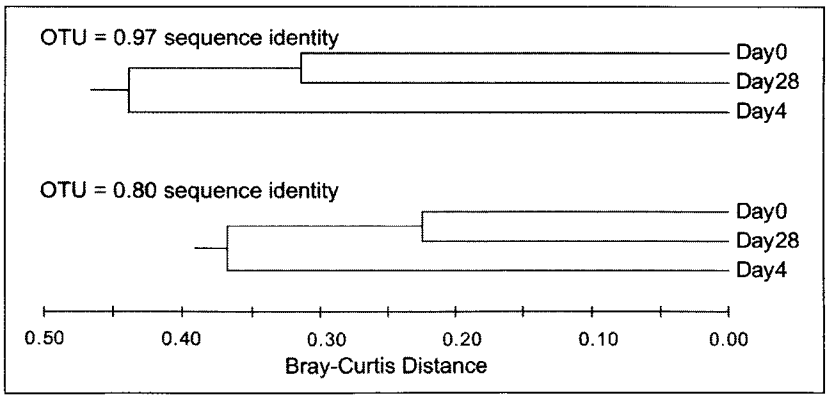

Figure 5. Dendrograms illustrating the relationships between the three 16S libraries from the antibiotic-associated diarrhea patient. Based on OTU assignment (at two levels, ≥97% and ≥80% sequence identity) the Bray-Curtis distance metric was calculated and then a UPGMA dendrogram constructed. For both OTU definitions, the Day 0 (pre-antibiotic) and Day 28 (2 weeks after antibiotic administration was stopped) communities were most similar.

been hypothesized that this reflects selection pressure to occupy specific ecologic niches, which in turns requires conserved sets of metabolic functions.[11]

Ecologic Statistical Analysis as a Means to Reduce Data Complexity

Analysis that involves clustering of communities also serves to reduce the complexity of large datasets. Although in the current example only three communities were compared, we have successfully used this form of analysis to compare 12 or more independent communities profiled by 16S clone library construction (unpublished data). Other investigators have employed forms of data reduction and clustering analysis to examine large sets 16S rRNA-encoding gene sequence data. Methods such as principal component analysis and partial least-squared regression can also detect distinct patterns within large datasets.[47] Eckburg and colleagues recently published a large 16S survey of human gut microbial diversity in which they used a method termed double principal coordinates analysis, to examine relationships between the colonic microbiota in different individuals and in different anatomic sites.[4,48] Ley and colleagues employed yet another method that compares multiple phylogenies (and therefore does not directly sequence divergence between clones, not does it require the assignment of sequences into specific OTUs) to examine the relationships between the cecal microbiota of mice that differed in leptin genotype.[10,49]

The existence of multiple methods for the community analysis resulting from the retrieval of 16S rRNA-encoding gene sequence data may suggest to some that it is difficult to obtain reliable "answers" from such data. However, although some investigators might wish to argue as to which type of analysis might be the "best," in our experience, the use of multiple methods of analysis provides complementary and (fortunately) noncontradictory information about the relationships between multiple microbial communities. In any case, it should be stressed that we view microbial community surveys in a manner analogous to exploratory microarray analysis. A great deal of data is generated, which needs to be simplified and once analyzed, the data generally do not provide a clear-cut answer, but allow the development of specific, testable hypotheses. The testing of such hypotheses may require additional non culture-based community analysis, but when it comes to testing hypotheses about microbial community function, culture-based analysis and biochemical analysis are likely to prove necessary. For example, our work on antibiotic-associated diarrhea has lead to a followup case-control study where we are attempting to correlate changes in the fecal microbial community with *C. difficile*-associated and non*C. difficile*-associated AAD. In order to do this, characterization of the specific *C. difficile* strains responsible for disease and actual determination of fecal short-chain fatty acid levels will be complementary to microbial community analysis. A recent metagenomic analysis of human feces suggested that there was an abundance of bacterial genes involved in the production of short-chain fatty acids, especially butyrate kinase, further suggesting that it is important to conduct experiments to test the functional significance of this finding.[38] It has been proposed that it is best to pursue a balanced approach, involving both large information-driven methods and classical microbial and biochemical methods to fully understand microbial community function.[50]

Summary

The complex community of microbes that inhabits the mammalian gut is part of an intricate ecosystem that involves the microbes, the host epithelium and the host immune system. The analysis of large, complex microbial communities has been revolutionized by the development of culture-independent methods that take advantage of the high throughput DNA sequence-driven techniques that made whole-genome analysis possible. The use of these techniques can provide a detailed determination of the structure of the gut microbial community and how this structure can be altered by disease states. An understanding of structure can lead to hypotheses about community function that can be tested by an integrated approach utilizing sequence-based techniques coupled with classical microbiologic, biochemical and immunologic analysis. It is likely that such studies will lead to a greater understanding of the relationship we have with the community of microbes that

inhabits our bodies. Hopefully, this understanding will lead to novel methods for the prevention and treatment of diseases that result from disturbances in this mutualistic symbiosis.

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CHAPTER 4

Effects of Microbiota on GI Health: Gnotobiotic Research

Robert Doug Wagner*

Abstract

The complex interactions between the GI tract microbiota and the immune system can be simplified for study using gnotobiotic animal models. The importance of cytokines, such as IFN-γ, TNF-α, TGF-β, Interleukin-2, IL-4 and IL-10 in the host response to intestinal bacteria has been evaluated using gnotobiotic studies. Gnotobiotic experiments with immunodeficient animals have revealed insights into the relationships between innate, cell-mediated and antibody-mediated immune system components in resistance to infectious microorganisms. The development and maturation of the immune system is dependent on the presence of some members of the intestinal microbiota. The commensal microorganisms, in turn, are dependent on the environment and nutrients provided by the GI tract of the host. Gnotobiotic studies are starting to reveal how the microbiota influences oral tolerance to dietary and commensal bacterial antigens. The immunomodulatory effects of microbiota and probiotics for inflammatory bowel diseases and the role of bacteria in their etiologies are being studied in gnotobiotic systems. Many aspects of the host interaction with the microbiota have been and will continue to be best addressed in gnotobiotic experimental models. This chapter reviews the contributions that gnotobiology has made to our understanding of the microbiota and host GI tract health.

Introduction

The large population of mixed bacteria, fungi and protozoa present in the GI tract from shortly after a person's birth throughout life is involved in the development and health of the gastrointestinal (GI) tract. Collectively, this mixed population of microorganisms has become known as the enteric microbiota and in numbers of cells it exceeds the population of the human cells of the GI tract. Multiple strains of hundreds of species of microorganisms live in a dynamic population in the microbiota and this complex ecosystem has been difficult to study experimentally. Several definitions need to be established for the remainder of this discussion. Gnotobiology is the experimental discipline of using living model systems in which the identities of all the organisms in the system are known. This is generally achieved by starting with germfree host animals that are then colonized with a defined microbiota. Germfree animals become "associated" or colonized with one or more kinds of microorganisms readily when orally inoculated.[1] Many experiments have been conducted on monoassociated animals that are gnotobiotic for a single strain of microbe living in or on them. Inadvertent colonization with microorganisms is called contamination, rather than association. Research animals that contain the natural microbiota adapted for residence in their wild counterparts are known as conventional animals and conventionalized animals are formerly germfree animals colonized with a wild-type microbiota. The use of gnotobiotic animal models

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has given us the control over variables needed to conduct experiments that have revealed much about GI tract development and health.

Germfree animals, once called "living test tubes",[1] are the basis of gnotobiotic technology and in the case of placental animals, they are derived by cesarean section and maintained by fostering with lactating germfree mothers. Gnotobiotic animals are housed in plastic isolators that receive air through high efficiency filters that exclude bacteria (Fig. 1A). Sterility is maintained in the isolator units by transferring materials into the isolators from steam-sterilized transfer boxes (Fig. 1B). The intestinal microbiota consists of a large number of commensal microorganisms that interact with the host in numerous ways. The commensal relationship is so intimate that the microbiota is involved in the proper development of host GI tract tissues. The closeness of the relationship between the microbiota and the host is exemplified by the role the microbiota play in the development and maintenance of the lymphoid tissues associated with the gastrointestinal tract. A relationship between inflammatory diseases and the GI tract microbiota has long been suspected and gnotobiotic research is starting to reveal valuable information about these health problems. Gnotobiotic studies open new insights into the colonization of bacteria in the microbiota and into the nature of infectious enteric diseases. A diverse microbiota resists addition of other microorganisms from persisting in the microbial population and this process often involves the host immune system. This chapter will highlight the contributions made by gnotobiotic research to understanding the dynamics of the GI tract microbiota and host defenses in health and disease. Table1 contains a list of the studies reviewed in the following sections.

Immunodeficient Gnotobiotic Models

Host defense at the interface of the GI tract epithelium and the lumen is a complex system consisting of cells from most of the immunological lineages and also of cells that are uniquely located in the gut associated lymphoid tissues (GALT). Gnotobiotic experiments allow simplification of the experimental models by eliminating the variables caused by bacterial growth, metabolism and infection. The use of immunodeficient animals can simplify the variables arising from the host system. Initially, immunodeficient gnotobiotic models using animals with natural immune system mutations were very helpful for studying GI tract infections. The natural mutant mice had vaguely defined genetic alterations that caused general debilitation of parts of the immune system. When gene targeting technology was applied to mice, transgenic animals deficient in the function of specific genes of the immune system could be tested. These models became outstanding tools for dissecting the immune response to microbiota organisms and invading pathogens or transient organisms in the GI tract.

Immunological mutant gnotobiotic mice were extensively studied in Edward Balish's laboratory using a model of GI tract colonization and infection by the dimorphic fungus *Candida albicans*. When BALB/c nude (*nu/nu*) mice having dysfunctional acquired immunity were monoassociated with *C. albicans*, their mucosal tissues (gastrointestinal and vaginal tracts) were infected with mostly yeast forms of the fungus.[2] Neonatal mice were clear of *C. albicans* in their GI tracts until 11-15 days after birth, suggesting protection from colonization by maternal antibodies. Adult and neonatal *nu/nu* mice were relatively resistant to mucosal candidiasis even without T-cell-mediated immunity. The data show the importance of innate immunity for protection from mucosal candidiasis. Other laboratories have also taken advantage of the gnotobiotic *nu/nu* mouse model to study translocation of bacteria in immunodeficient animals. Translocation is the passage of bacteria from the intestinal lumen across the intestinal epithelial cell monolayer to mesenteric lymph nodes and other internal organs. Translocation of *Bifidobacterium longum* occurred in monoassociated BALB/c nu/+ and *nu/nu* mice.[3] The *nu/nu* mice cleared *B. longum* from their internal organs only after reconstitution with immune cells from nu/+ mice. Immunoglobulin IgA was not produced in *nu/nu* mice, showing that translocation of the bacteria was controlled by T-cell-regulated humoral immunity.

The T-cell deficient nude mice were mated with "beige" mice deficient in innate phagocytic cell functions to yield a more immunodeficient bg/bg-*nu/nu* strain of mice. *Candida albicans*

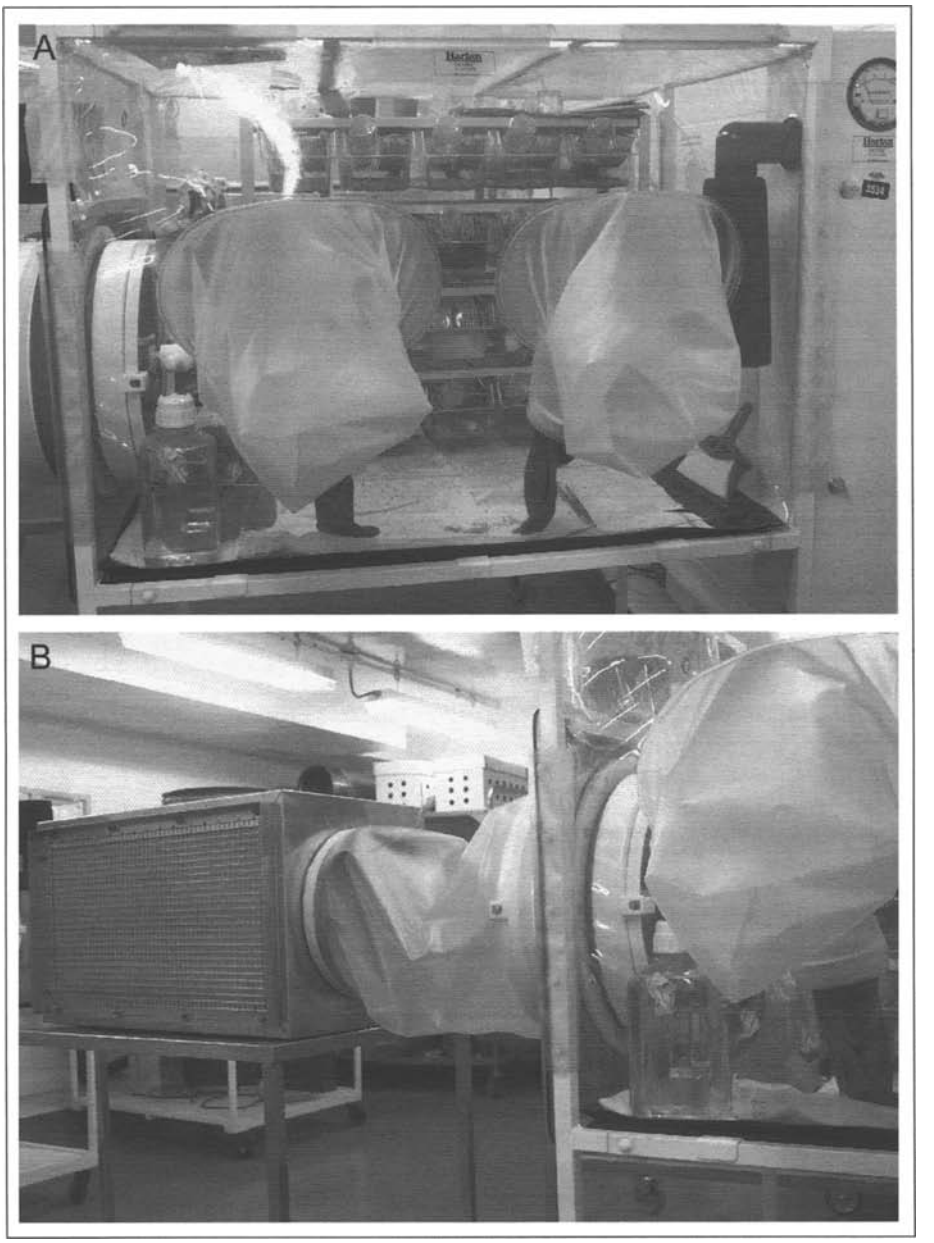

Figure 1. Flexible film isolators used in gnotobiotic experiments. A) A modern flexible film isolator with racked caging provides space to maintain rodents in germfree or gnotobiotic association. B) A steam-sterilized transfer box with a polyester film closure is used with a polyvinyl sleeve and chemical sterilizing agent to transfer supplies into the isolator.

monoassociation caused mucosal candidiasis in immunodeficient bg/bg-*nu/nu*, but not bg/bg, or heterozygous-nude bg/bg-nu/+ and nu/+ mice.[4] Enhanced susceptibility to orogastric candidiasis

Table 1. Gnotobiotic animal studies of GI tract health

Subject of Study	Species	Ref
Host responses to mucosal and systemic fungal infections	Immunodeficient mice	2-19
Translocation of bacteria	Immunodeficient mice	20
Effects on oral tolerance by ETEC *E. coli*	HMA mice	21
Thrombotic microangiopathy by ETEC *E. coli*	Mice	22
Gastric acid effects on *H. pylori* infection	Mice	23, 24
Antibody effects on translocation	Rats	25
Intestinal *E. coli* reduces inflammation in *S. enterica* infections	Mice	26
Anti-inflammatory effects of *S. enterica* vaccine	Pigs	27
Antimicrobial peptides in microbial antagonism	Rats	28
Bacterial induction of calprotectin	Pigs	29
Angiogenin 4 induction by *B. thetaiotaomicron*	Mice	30
Probiotic protection of immunodeficient mice	Immunodeficient mice	31, 32
Probiotic protection with heat-killed bacteria	Immunodeficient mice	33
Immunostimulation by heat-killed lactobacilli	Mice	34
Anti-inflammatory effects of probiotics	Rats	35
Probiotics induce oral tolerance	Mice	36
Probiotic suppression of *S. enterica* infection	Mice	38
Enhanced antibody response to *C. albicans* by probiotic bacteria	Immunodeficient mice	39
Mucous production induced by microbiota	Mice, Rats	40
Microbiota induced MALT development	Pig	41
Microbiota promotes development of Peyer's patches	Mice, Rats	42, 43
H. muridarum induced colitis model of IBD	Immunodeficient mice	48
IgA secreting cell induction by SFB	Mice	49
Colitis model induced by SFB + *B. vulgatus* + dextran sulfate	Immunodeficient mice	50, 52
Microbiota suppress colitis-associated T-cells	Immunodeficient mice	51
Specific bacteria induce colitis in IL-2$^{-/-}$ mice	Immunodeficient mice	54

Abbreviations: Ref. = references, ETEC = enterotoxigenic *E. coli*, HMA = human microbiota-associated, *E. coli* = *Escherichia coli*, *H. pylori* = *Helicobacter pylori*, *S. enterica* = *Salmonella enterica*, *B. thetaiotaomicron* = *Bacteroides thetaiotaomicron*, *C. albicans* = *Candida albicans*, *H. muridarum* = *Helicobacter muridarum*, *B. vulgatus* = *Bacteroides vulgatus*, IBD = inflammatory bowel disease, SFB = segmented filamentous bacteria

required reduction of both innate and acquired immune functions. *Candida albicans* monoassociation of bg/bg-*nu/nu* mice led to lethal systemic candidiasis of endogenous origin.[5] The model provided a means to study the course of lethal translocation of *C. albicans* from the GI tract to internal organs. Previously, systemic candidiasis was modeled in mice by tail vein injection of large

numbers of fungal cells. *Candida albicans* monoassociated bg/bg-nu/+ mice developed specific T-cell and antibody responses, which showed that acquired immunity was sufficient to protect susceptible mice from candidiasis even when they had dysfunctional innate immunity. Regardless of the severe immunodeficiency imparted by the beige and nude mutations, *C. albicans* monoassociated bg/bg, *nu/nu* and bg/bg-*nu/nu* mice were all resistant to vaginal candidiasis.[6]

Another natural mutation in mice is severe combined immunodeficiency (SCID), which is characterized by a lack of functions associated with acquired immunity. Intraperitoneal injection of cyclophosphamide (phagocytosis inhibitor) increased susceptibility of *C. albicans* monoassociated SCID mice to mucosal candidiasis, showing that innate immunity is adequate for protection and phagocytic neutrophilic leukocytes are important for mucosal resistance to *C. albicans*.[7]

Animal models ablated of specific types of immune cells were made by treatments with monoclonal antibodies. Mice with the "beige" defect in phagocytic cell function and heterozygous for the "nude" defect in T-cell function (bg/bg nu/+), but not homozygotes (bg/bg-*nu/nu*), had $CD4^+$ T-cells in Peyer's patches and spleens with interleukin-2 (IL-2) production and proliferative responses to *C. albicans* antigens.[8] The bg/bg-nu/+ mice became more susceptible to mucosal *C. albicans* infections when they were injected with monoclonal antibodies to the CD4 lymphocyte antigen. Intraperitoneal injections of monoclonal antibodies to IL-2 and interferon-γ (IFN-γ) did not reduce resistance, suggesting that redundant mechanisms of protection were not dependent on the cytokines. Treatment with IL-2 and IFN-γ did not enhance resistance of the bg/bg-*nu/nu* mice, confirming that they did not have T-cells that could be activated by the cytokines. IFN-γ is important for activation of cytotoxic T-cells, which account for much of the cell-mediated immunity that protects immunocompetent mice from *C. albicans* infection. The inability to mount acquired immune responses in *nu/nu* mice was also confirmed by the inability to develop lymphocyte proliferation and footpad swelling responses to antigens.[9] The value of monoclonal antibody ablation of specific cytokines was also illustrated using the gnotobiotic SCID mice. Treatment of *C. albicans* monoassociated CB-17 SCID mice with poly (I.C), which induces interferon production, increased susceptibility to experimental (intravenous challenge) and systemic candidiasis of endogenous (oral challenge) origin.[10] The susceptibility to candidiasis was reversed by in vivo neutralization of IFN-α, β and γ with monoclonal antibodies.

When gene-targeted specific "knockout" mice became available, gnotobiotic candidiasis susceptibility studies were conducted on them. The importance of several regulatory cytokines in host responses to pathogens was evaluated with specific gene-targeted knockout mice. Germfree Interleukin (IL)-10 and IL-4 knockout mice were as resistant to mucosal candidiasis after monoassociation with *C. albicans* as were immunocompetent controls.[11] The IL-10 knockout mice were more resistant and the IL-4 knockout mice were more susceptible to experimental systemic candidiasis than control mice. This study revealed that levels of expression of IL-10 and IL-4 are more important in resistance to systemic candidiasis than in mucosal candidiasis. Interferon-γ knockout mice monoassociated with *C. albicans* developed severe mucosal candidiasis, had Th_2-type IgG_1 serum antibody responses and were more susceptible to intravenous challenge systemic candidiasis than normal mice.[12] When the murine homolog of the IL-8 receptor gene of humans was disabled in BALB/c mice,[13] germfree BALB/c IL-$8Rh^{-/-}$ mice were more susceptible than immunocompetent control mice to oral or systemic challenge with *C. albicans*. Reduced responses by polymorphonuclear cells were detected that shows the importance of IL-8Rh gene expression in protection of mice from candidiasis.

Gene-targeted knockouts of immunological cell types were also studied under gnotobiotic conditions. The J_HD strain of B-cell knockout mice was as resistant to orogastric and disseminated candidiasis of endogenous origin (oral challenge) as immunocompetent control mice.[14] The J_HD mice were also resistant to systemic candidiasis initiated by intravenous challenge. This shows that innate and T-cell mediated immunity protects mice from candidiasis independently of humoral immunity. Mice with the β_2-microglobulin gene knocked out are deficient in major histocompatibility class I antigen expression and in $TCR\alpha\beta^+$ T-cells.[15] Although they could mount an antibody response after monoassociation with *C. albicans*, they were susceptible to systemic candidiasis of

endogenous origin. They were, however, resistant to intravenous challenge systemic candidiasis. Therefore, the data suggest that TCR$\alpha\beta^+$ CD8$\alpha\beta^+$ T-cells are necessary for development of protective immunity to *C. albicans* by oral inoculation.

Some mice are made immunodeficient by insertion mutagenesis of human genes into their murine analogs. Insertion of homologous human genes into the DNA of rodents often abrogates the function of the rodent gene, as shown in a study of transgenic rats with human HLA-B27 and β_2-microglobulin that were very susceptible to infection by *Listeria monocytogenes* compared to normal rats.[16] Another mouse model was made that abrogated T-cell and NK cell functions by introduction of a human CD3ε gene into the mouse genome. These mice are typical of many transgenic strains that fail to thrive in a conventional environment, but thrive under germfree conditions (Fig. 2). Transgenic epsilon 26 (Tgε26) mice with defective CD3ε^- T-cells (lack T-cells and NK cells expressing CD3 accessory molecules) were very susceptible to oroesophageal candidiasis when monoassociated with *C. albicans*.[17] The mice were resistant to intravenous challenge systemic candidiasis showing the importance of NK and T-cells for protection of mice from *C. albicans* introduced through the gastrointestinal tract. β-Defensin mRNA expression was compared in *C. albicans* monoassociated C57BL/6 and Tgε26 mice.[18] The transgenic mice, though deficient in T-cells, were capable of induction of β-defensins 1, 3 and 4 when monoassociated with *C. albicans*. The immunocompetent C57BL/6 mice had more β-defensin 4 expressed in gastric tissues than the transgenic mice.

The Tgε26 mouse model was also used to study activation of *C. albicans* virulence-associated genes.[19] Expression of secretory aspartyl proteinase and phospholipase B genes by *C. albicans* in immunocompetent and immunodeficient mice was measured to ascertain if host immune status affects expression of these virulence factors. In transgenic mice that are deficient in T and NK cell activity and in mice that are deficient in nitric oxide and phagocyte oxidase production, no

Figure 2. Germfree conditions support immunodeficient mice that fail to thrive under conventional conditions. The mouse at the top is a one year old female Tgε26 human microbiota-associated mouse that displays stunted growth, ruffled coat and a tumor below the jaw. The lower mouse is an example of a healthier one year old female Tgε26 germfree mouse.

differences in virulence gene transcription were observed. These specific gnotobiotic models improved our understanding of the complex interactions of various compartments of the immune system in host defense against *C. albicans* infections.

Immunological Effects of GI Tract Infections in Gnotobiotic Animals

Systemic infections may often start at the point that infectious microbes translocate from the GI tract to the internal tissues. Translocation rates of enteric bacteria were measured in T-cell-deficient germfree BALB/c "nude" ***nu/nu*** mice.[20] Facultative Gram-negative bacteria translocated at greater rates than facultative Gram-positive bacteria, which translocated more than obligate anaerobic *Bacteroides* spp., *Fusobacterium* spp. and *Bifidobacterium* spp. These results suggest that the normal microbiota, which consists mostly of obligate anaerobes, has the lowest rate of translocation and requires the least degree of control by the mucosal immune system. A change in the microbiota favoring growth of Gram-negative bacteria would probably cause increased bacterial translocation.

Gnotobiotic studies have revealed insights into the interactions of the microbiota and virulent *Escherichia coli*, especially enterotoxigenic (ETEC) *E. coli*. Immunological tolerance can become established to many proteins when they are ingested by mice with intestinal microbiota. The heat-labile enterotoxin of *E. coli* can abrogate oral tolerance to some proteins.[21] In the study, human microbiota associated mice had significantly suppressed IgG, IgG1, IgG2a and IgE responses to ovalbumin that was co-introduced with enterotoxin to the mice. Thus, the donor human microbiota contained bacteria that could suppress the effect of enterotoxin. Conventional mice were also hypo-responsive to ovalbumin but *E. coli* monoassociated mice were responsive to it. These results imply that some, but not all kinds of bacteria can promote oral tolerance even in the presence of enterotoxin. Early association with a complete microbiota during postnatal development is important for tolerance induction. Germfree piglets were orally infected with Shiga toxin 2-producing *E. coli* O157:H7 or O26:H11, which caused them to exhibit symptoms of thrombotic microangiopathy in the kidneys, analogous to the human disease.[22] This is the first report from this animal model of the human pathophysiology of enterohemorrhagic *E. coli* infections, as conventional swine are not known to exhibit this disease.

A complete microbiota complicates the study of individual bacteria in vivo, but complications caused by experimental animal behavior needs to be considered also. Germfree mice were used in a study because the coprophagic nature of conventional mice keep their stomachs colonized with a diverse microbiota that prevents a clear observation of tropism by individual species like *Helicobacter pylori* into specific ecological niches in the gastric mucosa.[23] The authors of the latter paper used FVB/N mice, which express a fragment of diphtheria toxin A in their H+/K+ ATPase gene, causing ablation of gastric parietal cells. The Hp1 strain was adherent to the junction between the fore-stomach and zymogenic region of the normal control mouse stomach, analogous to the cardial region of the human stomach epithelium. The bacteria avoided the antrum and zymogenic regions of the stomach. The location had favorable conditions of pH and low enzyme levels and also the presence of neuraminic acid-α2,3-galactose-β1,4-glycans, which are bound by bacterial adhesins. The stomachs of infected mice had diffuse gastritis and lymphoid aggregates. The lymphoid aggregates contained elevated levels of expression of genes associated with inflammatory T and B-cells, NK cells, macrophages and dendritic cells. The transgenic mice had more generalized infections of the stomach epithelium, showing that parietal cell activity discourages *H. pylori* Hp1 growth in normal mice. Another study illustrated how acid-producing parietal cells are important in *H. pylori* pathogenesis. Gnotobiotic transgenic mice deficient in gastric acid production that mimic the chronic atrophic gastritis disease monoassociated with *H. pylori* were tested for the level of transmission of *H. pylori* from infected to uninfected animals.[24] Some transmission of *H. pylori* occurred between mice with achlorhydria supporting the hypothesis that the low acid producing state of children may make them more susceptible to infection.

Gnotobiotic Studies of Microbial Antagonism

The GI tract microbiota has a profound effect on one's susceptibility to infection by pathogenic organisms and viruses. When the microbiota inhibits the colonization, growth, or virulence of a pathogen it is exerting microbial antagonism. Several mechanisms appear to account for the effect of microbial antagonism, also called colonization resistance or the "barrier effect". This section will focus on the mechanisms of microbial antagonism associated with the immune system.

Many studies have been conducted to determine how the microbiota interacts with the host mucosal immune system to prevent pathogens from colonizing the GI tract. Obligate anaerobes appear to reduce translocation of facultative anaerobes in the intestines.[25] Induction of specific and cross-reactive antibodies may be involved in this process. A gnotobiotic study used rats associated with *E. coli* and *Peptostreptococcus sp.* that had reduced bacterial translocation of *E. coli* compared to *E. coli* monoassociated rats.[25] Titers of antibodies to the *E. coli* and cross-reactive with the other species, were also increased by the presence of *Peptostreptococcus sp.* in the rats.

Collateral damage from inflammatory responses increases the severity of GI tract diseases. The presence of some bacteria in the GI tract can modulate the severity of an inflammatory response. *Escherichia coli* monoassociated mice survived better than germfree mice challenged with *Salmonella enterica* Serovar Typhimurium.[26] The *E. coli* monoassociated mice had more intact mucosal surfaces and fewer inflammatory signs, such as edema, cellular infiltration and hyperemia. The numbers of *S. enterica* cells in the intestines were not decreased, indicating that reduced inflammation was the principle reason for increased survival of the gnotobiotic mice. Germfree pigs and pigs monoassociated with an avirulent strain of *Salmonella sp.* were challenged with virulent *S. enterica* serovar Typhimurium, disease was scored and ileal cytokine production was measured.[27] The presence of the avirulent strain alleviated severe systemic salmonellosis, induced the ileal production of IL-8 and reduced the production of IL-1β, IL-10 and TNF-α in response to the virulent strain. These cytokine profiles suggest that the host response to the avirulent strain was less inflammatory, inducing less collateral tissue damage.

The production of bacteriocins and other antimicrobial peptides by microbiota bacteria are also part of the microbial antagonism phenomenon. A trypsin-dependent substance produced by Peptostreptococcus sp. in monoassociated rats inhibited intestinal colonization by *Clostridium perfringens*.[28] Antimicrobial peptides are also produced by the host and they may be stimulated by the microbiota. For example, differences in plasma calprotectin levels were compared between germfree, *E. coli* O86, *E. coli* strain Nissle 1917 and enteropathogenic *E. coli* O55 monoassociated pigs.[29] The presence of *E. coli* Nissle 1917 increased small intestine luminal calprotectin levels and reduced the severity of disease, suggesting that the therapeutic effects of *E. coli* Nissle 1917 on inflammatory bowel disease may occur by intestinal mucosal immunostimulation with calprotectin secretion. Another class of antimicrobial peptides is the angiogenins. Paneth cells from *Bacteroides thetaiotaomicron* monoassociated mice produce angiogenin 4, which is bactericidal to *Enterococcus faecalis* and *Listeria monocytogenes*, but not to *Listeria innocua*, *E. coli* K12, or *B. thetaiotaomicron*.[30]

The concept of microbial antagonism has made it to the marketplace in the form of "probiotics". Probiotics are live microbial dietary supplements ingested with the intention of increased microbial antagonism and other health benefits. Probiotics could be very useful adjuncts to other therapies for protection of immunodeficient patients. The safety of these bacteria for the immunodeficient consumer needs to be established. We conducted a series of experiments in gnotobiotic immunodeficient mice to evaluate safety of individual species of probiotic bacteria. We also investigated the capacity of the probiotics to protect immunodeficient mice from candidiasis. Immunodeficient bg/bg-nu/+ and bg/bg-*nu/nu* mice were monoassociated with *Lactobacillus acidophilus*, *Lactobacillus reuteri*, *Bifidobacterium* (formerly *animalis*) *lactis* Bb-12, or *Lactobacillus* (formerly *casei*) *rhamnosus* GG.[31] The mice were orally challenged with *C. albicans*, which infects the tongue, esophagus and stomach of these mice. Survival and incidence of disseminated candidiasis of endogenous origin were significantly reduced in the immunodeficient mice that were colonized with any of the probiotic bacteria. The experiments show that thymic, extrathymic and non-immunological mechanisms are involved in resistance of mice to *C. albicans* infections.

We have found that safety and efficacy of probiotic bacteria species vary between strains. Neonatal immunodeficient bg/bg-*nu/nu* mice were susceptible to mortality from *L. reuteri*, or *L. rhamnosus* GG under gnotobiotic conditions.[32] Neonatal mice monoassociated with *L. acidophilus* or *B. lactis* Bb-12 did not succumb. Since there is some risk of infection by viable probiotic bacteria in immunodeficient hosts, the ability of heat-killed *L. acidophilus* or *L. rhamnosus* GG to protect immunodeficient bg/bg-*nu/nu* and bg/bg-nu/+ mice from oral *C. albicans* challenge was investigated.[33] Severity of orogastric candidiasis lesions was reduced in both strains of mice by both strains of heat-killed lactobacilli. Both preparations protected bg/bg-nu/+ mice from disseminated candidiasis up to 4 weeks after challenge. The heat-killed *L. acidophilus*, but not heat-killed *L. rhamnosus* GG inhibited disseminated candidiasis in bg/bg-*nu/nu* mice for 2 weeks after challenge. Another study using immunocompetent mice showed that heat-killed *L. acidophilus* monoassociated mice could clear intravenously injected *E. coli* from internal organs better than germfree mice.[34] This is further evidence that probiotic bacteria can act as immunostimulants that impart resistance characteristics against pathogenic bacteria and the stimulation does not necessarily require viable bacteria.

Probiotic bacteria appear to have anti-inflammatory or immunomodulating properties as well as immunostimulatory properties. In a recent study, rats were monoassociated with *Bifidobacterium adolescentis* or *Bacteroides thetaiotaomicron* and fecal IgA, IgG and secretory IgA (sIgA) were measured by enzyme-linked immunosorbent assay.[35] *Bifidobacterium adolescentis* did not induce serum immunoglobulin production but fecal sIgA was induced. The immunoglobulin response to *B. thetaiotaomicron* was reduced by the presence of the probiotic *B. adolescentis*. This immunomodulating effect of probiotic bacteria was illustrated further by their ability to induce oral tolerance to antigen challenge. Mice monoassociated with *Lactobacillus paracasei*, *Lactobacillus johnsonii*, or *B. lactis* Bb-12 were assessed for tolerance induction to bovine β-lactoglobulin in whey proteins fed to mice injected subcutaneously with β-lactoglobulin.[36] Humoral and cellular immune responses (serum IgE, IgG1, IgG2a and phytohemagglutinin-induced splenocyte proliferation) were suppressed more in conventional microbiota-associated mice than in the monoassociated mice. The monoassociated mice had more suppression of immune responses than the germfree mice. *Lactobacillus paracasei* suppressed immune responses (induced oral tolerance) better than *B. lactis* Bb-12 or *L. johnsonii*. Monoassociation of mice with *L. acidophilus* or *L. casei* doubled the numbers of intestinal intraepithelial lymphocytes (IEL).[37] However, the phenotypes of IEL after monoassociation of the mice were Thy 1.2^- $CD3^+$ $CD4^-$ $CD8^+$, the same as in the germfree mice, showing that the bacteria did not change the activation status of the IEL. The failure of these *Lactobacillus spp.* to induce expansion of Thy 1.2^+ cells may be an indication of how the immune response may be modulated by the presence of these bacteria. Perhaps, expansion of Thy 1.2^- T-cells inhibits expansion of more reactive Thy 1.2^+ T. cells. *Bifidobacterium longum* monoassociated mice and conventional mice were challenged with *S. enterica* serovar Typhimurium and 40% of monoassociated mice survived when germfree mice did not.[38] A significantly greater number of conventional mice fed *B. longum* in milk survived 28 days after *S. enterica* challenge than conventional mice not fed the probiotic. Protection was not afforded by reduction of *S. enterica* populations, but probably by reduction of inflammation.

One way gnotobiotic experiments have shown that probiotic bacteria modulate immune responses to pathogens is by increased antibody responses, or changes in production of specific antibody isotypes. Probiotic bacteria *L. acidophilus*, *L. reuteri*, *L. rhamnosus* GG and *Bifidobacterium infantis* were monoassociated into immunodeficient bg/bg-*nu/nu* and bg/bg-nu/+ mice.[39] Specific antibody responses to bacterial antigens and *C. albicans* antigens were detected after oral challenge with *C. albicans*. The presence of *B. infantis* enhanced IgG1, IgG2a and IgA production to *C. albicans* antigens. Some IgG1 and IgG2a production was induced by the probiotic bacteria in bg/bg-*nu/nu* mice. The study shows that enteric bacteria can affect specific and nonspecific antibody production to pathogens like *C. albicans*.

Microbiota Effects on Gut Associated Lymphoid Tissue Architecture

The architecture of the GI tract is complex and specialized for responding to microbes and antigens in the gut. Figure 3 is an illustration of several parts of the GI tract villus structure and the Peyer's patches of the GALT that will help us navigate through the discussion in this section. Mucous production by intestinal goblet cells is important for GI tract health. The mucous provides protection for the epithelial cell layer of the GI tract and also provides an environment for the microbiota. Germfree rodents produce fewer and smaller goblet cells than rodents with conventional microbiota, an indication that the microbiota induces development of intestinal architecture.[40] Increased mucous production occurred in conventional mice and rats compared with germfree mice and rats. The microbiota has an intimate relationship with the host, which has adapted to respond to the microbial presence by adjustment of the GI tract architecture in the form of increased mucous production. This arrangement is a classic example of a commensal arrangement. The microbiota receives an ideal ecological niche in mucous and the host gets protection from transient bacteria in the gut.

Gnotobiotic pigs were used as a model of mucosal-associated lymphoid tissues (MALT) development in humans.[41] Histology of fetuses showed that MALT is developed in the pigs at birth. Fully developed MALT in the stomach was observed in neonatal pigs. Pigs don't have maternal

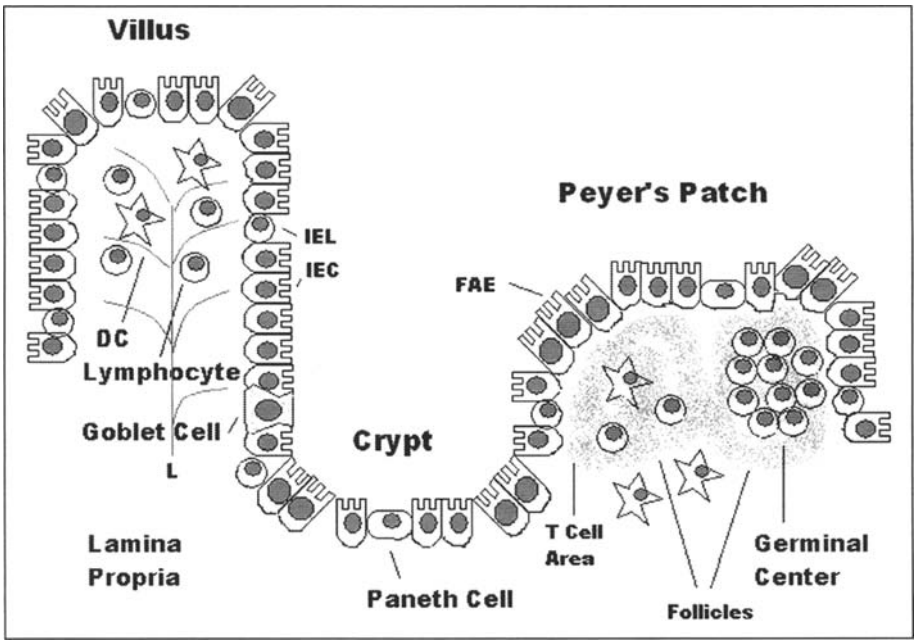

Figure 3. Gut-associated lymphoid tissues at the GI tract lumen. The intestinal villus consists of a layer of intestinal epithelial cells (IEC) and intraepithelial lymphocytes (IEL) surrounding a core called the lamina propria that contains lymphocytes, dendritic cells (DC) and lymphatic sinuses (L). A crypt at the base of a villus contains IEC, IEL, mucous-secreting goblet cells and paneth cells that secrete antimicrobial peptides. The Peyer's patches are specialized lymphoid regions of the intestinal epithelium. Their luminal surface is the follicular-associated epithelium (FAE), which contains IEC, IEL and specialized "M" cells that sample antigens from the intestinal lumen. Follicles form beneath the FAE that contain accumulations of lymphocytes. Follicles containing expanding clones of B-cells are germinal centers. T cell areas contain T-cells and mononuclear cells including dendritic cells. The presence of bacteria in the gut lumen increases the numbers of IEL and goblet cells in the epithelial monolayer, the numbers of dendritic cells in the intestinal lamina propria and germinal centers in the Peyer's patches.

immunoglobulin transfer through the placenta, so prenatal development of mucosal defenses is necessary for the survival of these animals. By the time of birth, pigs have all the necessary components in their stomach GALT for development of an immune response. The stomach GALT is not as distinctly structured as the GALT of the small intestines. The stomach GALT in the fetal pigs contains follicle-like lymphoid aggregates, diffuse mononuclear infiltrates analogous to the paracortex (T-cell areas) of more organized lymphoid tissues and intraepithelial lymphocytes that execute host responses to microbes.

As the first line of defense against invading pathogens, the immunological tissues of the GI tract are extensive and unique in cell composition, tissue structure and function. Gnotobiotic studies have revealed roles for the GI tract microbiota in the development and maintenance of mucosal immune tissues. The small intestine contains specialized lymphoid tissue structures called Peyer's patches that are sites of accumulation and activation of lymphocytes that respond to antigens in the GI tract. Gnotobiotic experiments have shown that germinal center reactions (observation of lymphocyte accumulations in Peyer's patches) and specific IgA production occur in mice monoassociated with microbiota-derived species, like *Morganella morganii*.[42] The process of conventionalization of germfree rats is associated with lymphocyte accumulation in follicle-associated epithelial regions of the Peyer's patches.[43] Before the lymphocytes accumulate, immature $CD4^+$ $CD86^-$ dendritic cells are found in the FAE of the Peyer's patches. In germfree rats, the mature $CD86^+$ dendritic cells are found in interfollicular zones, but then they disappear after conventionalization. In the latter study, organized follicular germinal center reactions did not occur in response to microbiota antigens, even though IgA specificity changed, showing that a T-cell-independent response coincides with a T-cell-dependent one in the presence of a commensal microbiota. Conventionalization causes dendritic cells to disappear from FAE of Peyer's patches, which are replaced by mostly B and T-cells. Conventional intraepithelial B-cells are $CD86^+$ and FAE B-cells do not express Bcl-2, but follicular mantles contain Bcl-2^+ B-cells. These results reveal how the composition of Peyer's patches changes in response to intimate association with the microbiota.

Comparing germfree and conventional mice, it was found that the microbiota induces IgA production independently of T-cell help and this IgA induction does not influence serum IgG levels.[44] This shows that accumulation of localized mucosal immune responses may not trigger systemic antibody responses.

Gnotobiotic studies have revealed that dendritic cells in the lamina propria of Peyer's patches and mesenteric lymph nodes are involved in tolerance to self antigens.[45] Refer to Figure 4 for an illustration of the structure of mesenteric lymph nodes. Dendritic cells linked to self-tolerance generally produce nonspecific esterase (NSE) and these cells are present in interfollicular and T-cell areas of the mesenteric lymph nodes and lamina propria of Peyer's patches of conventional rats. Dendritic cells linked to self-tolerance (nonspecific esterase-producing dendritic cells) are present in interfollicular and T-cell areas of the lamina propria of Peyer's patches and mesenteric lymph nodes of conventional rats. Dendritic cells are found only in the ileum and T-cell areas of mesenteric lymph nodes of germfree rats. The presence of a microbiota is not necessary for migration of these dendritic cells into intestinal lymphoid tissues, suggesting that self-tolerance mechanisms are not affected much by the microbiota. Apparently, the NSE^+ dendritic cells function in germfree or associated rats is to phagocytose apoptotic epithelial cells and present their antigens to T-cells in secondary lymphoid tissues for maintenance of self-tolerance.

Regulation of oral tolerance appears to be centralized to the Peyer's patches. Oral tolerance induction was compared in germfree, conventional, or *B. infantis*, *E. coli*, *C. perfringens*, or *Staphylococcus aureus* monoassociated mice.[46] Germfree and *C. perfringens* or *S. aureus* monoassociated mice had fewer Peyer's patches in their small intestines than conventional or *B. infantis* or *E. coli* monoassociated mice and they had a corresponding increase of IgG_1 production in response to ovalbumin. The presence of T-cells in Peyer's patches of conventional and *B. infantis* or *E. coli* monoassociated mice was needed to induce oral tolerance to the ovalbumin antigen. Given that germfree mice appear to lack the ability to induce oral tolerance, these results suggest

that the presence of certain bacteria in the microbiota are essential for the recruitment of T-cells to the Peyer's patches that are involved in oral tolerance induction.

Based on conventional animal studies, it was believed that the B1 class of B-cells was responsible for production of much of the IgA secreted into the GI tract. Gnotobiotic studies with *M. morganii*, *Bacteroides distasonis* or segmented filamentous bacteria (SFB) monoassociated mice with B1 and B2 cell allotypic chimeras proved that B2 cells produce most of the IgA response to the microbiota.[47] The gnotobiotic studies with genetically modified mice revealed insights that were not gained from conventional animal studies. Individual species of gut-colonizing bacteria induce activation of germinal center reactions in Peyer's patches and increased IgA to specific microbial antigens.[48] B1 and B2 cells, along with helper T-cells are required for these responses. *Helicobacter muridarum*-monoassociated SCID mice develop an inflammatory bowel disease-like wasting syndrome by infiltration of the large intestine by activated CD_4^+ T-cells. Regulatory Tr_1 cells can ameliorate the disease, as seen when Tr1 cells are adoptively transferred from *H. muridarum*-monoassociated immunocompetent mice to germfree SCID mice, which activates symptoms of *H. muridarum*-induced inflammatory bowel disease. This work is revealing new information about self-tolerance and regulation of mucosal inflammatory responses.

Some bacteria species in the microbiota affect the structure of intestinal lymphoid tissues more than others. Mice monoassociated with SFB were compared with germfree and conventional microbiota mice for numbers of lymphocytes in the lamina propria of ileum and cecum.[49] The SFB monoassociated mice had more lymphocytes and IgA secreting cells in their lamina propria than the germfree mice.

Inflammatory Responses to the Microbiota

Microbiota bacteria have long been suspected of being involved in the etiologies of inflammatory bowel diseases. The complexity of the normal microbiota has made it difficult to determine which bacteria should receive more attention for study. Gnotobiotic studies are starting to reveal

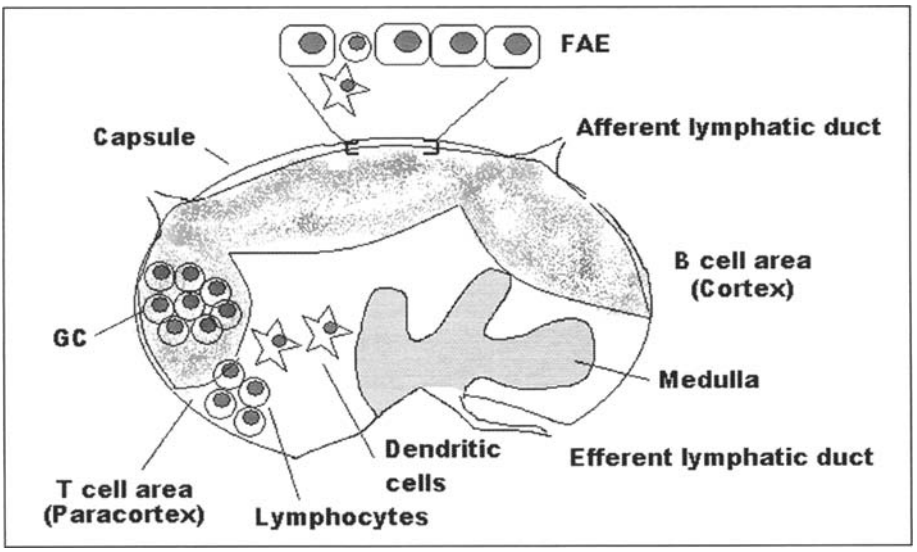

Figure 4. Structure of a mesenteric lymph node. A capsule surrounds the follicular-associated epithelium (FAE) of the lymph node. Afferent and efferent lymphatic ducts penetrate the capsule. The B-cell area, or cortex, contains germinal centers. T-cells accumulate amid dendritic cells and other mononuclear cells in the paracortex. The medulla is a sinus that drains the lymph node into the efferent lymphatic ducts. The presence of bacteria in the gut lumen increases the numbers of germinal centers and the numbers of IEL and dendritic cells in the FAE.

insights into the microbial component of these diseases. *Bacteroides vulgatus* and SFB-associated mice fed *dextran sulfate* develop an intestinal inflammatory response similar to ulcerative colitis.[50] When these mice were coassociated with *Bifidobacterium breve*, *Bifidobacterium catenulatum* and *B. longum* isolated from patients with ulcerative colitis, the severity of inflammation indicated by myleoperoxidae, occult blood scores and intestinal IgA leakage markers was reduced. Activated T-cells in bone marrow transplanted into germfree Tgε26 immunodeficient (T and NK cell functions) mice did not elicit colitis, but it did when the bone marrow cells were transplanted into conventional Tgε26 mice.[51] When fed dextran sulfate, conventional SCID mice, like immunocompetent BALB/c mice had intestinal inflammation similar to human inflammatory bowel diseases.[52] Germfree SCID mice did not develop intestinal inflammation after dextran sulfate feeding. This showed that the intestinal microbiota is involved in the development of inflammatory bowel disease induced by dextran sulfate in mice. Being immunodeficient for T and B-cells, the SCID mice show that the inflammatory response to dextran sulfate is part of the innate immune system, not the acquired immune system. Food proteins like wheat gliadins, which may be mimicked by dextran sulfate, activate NF-κB-regulated cytokine production, which activates and extends chronic inflammation. Additional cellular components of enteric bacteria appear to also be involved in this process.

The presence of a complex microbiota is usually associated with reduced inflammatory responses to GI tract pathogens. Although germfree mice have distinctly different GI morphology and immunological characteristics from streptomycin-treated conventional mice, the pathogenesis and early inflammatory response were quite similar after *Salmonella* sp. challenge.[53] However, cecal epithelial damage was increased and recovery was diminished in the germfree mice. Immune system defects can lead to situations where the microbiota can exacerbate inflammatory responses. Interleukin-$2^{-/-}$ mice had no colitis in the germfree state, but acquired colitis after conventionalization.[54] Colitis was induced in IL-$2^{-/-}$ mice after monoassociation with *E. coli* mpk, but not after monoassociation with *E. coli* Nissle 1917 or *B. vulgatus*. There were increases in mRNA production of IFN-γ, TNF-α, CD14 and IL-10 associated with monoassociation by *E. coli* mpk in IL-$2^{-/-}$ mice. These results suggest that *E. coli* strains have variable abilities to modulate inflammatory responses in immunocompromised hosts.

New Directions for Gnotobiotic Studies of the Microbiota and Immunity

Gnotobiotic animal models have proven their worth in studies of the complicated interactions of the GI tract microbiota and mucosal immune system. Interactions of defined microbiota with immune systems that are deficient in specific genes are the current state of the science. The unique mucosal immune system is yielding to this technology answers to many questions about regulation of the host response to commensal microorganisms and how they affect the host. Inquiries into the contributions of viruses, protozoa and fungi to microbiota-host interactions have only begun and are a new frontier for gnotobiotic research. Future research will continue to discover the mechanisms behind oral tolerance regulation by the microbiota and how that system can be applied for deliberate immunoregulation of systemic immunity. The mechanisms that limit translocation of microorganisms from the GI tract still need to be worked out in gnotobiotic studies. Given the diverse and complex nature of the intestinal microbiota, further advances in understanding and development of experimental techniques in microbial ecology will be dependant on gnotobiology. The mechanisms of probiotic effects on host immunity still need to be determined and gnotobiology will expedite these future studies. In spite of the expense, requirements for skilled workers and time needed to execute gnotobiotic experiments, they will be required in all the aforementioned disciplines by the continued complexity of the commensal relationships of microbiota and host.

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CHAPTER 5

Positive Interactions with the Microbiota: Probiotics

Marko Kalliomäki,* Seppo Salminen and Erika Isolauri

Abstract

Rigorous research in the field of probiotics is a fairly new phenomenon although first reports about beneficial effects of specific gut bacteria on human health originated already a century ago. A prerequisite for such a scrutiny has been a definition of criteria for probiotics. Recently, novel molecular technologies have characterized both potential targets of probiotic action, like gut microbiota and established and candidate probiotic strains in more detail. We thus propose here revised criteria for selection of probiotics. In addition to several promising clinical studies e.g., in the prevention and treatment of atopic eczema, certain probiotics have been found to maintain intestinal equilibrium by enhancing the gut mucosal barrier via manipulation of expression of several their own and the host's genes. Introduction of genetic engineering has provided advanced tools to amend probiotics' properties in the fight against different inflammatory conditions.

Introduction

The first reports about health-promoting effects of lactobacilli and bifidobacteria, currently the two most often used genera in probiotic research, on human health were published a century ago. A French scientist Tissier recommended large doses of bifidobacteria for treatment of infantile diarrhoea and a Nobel laureate Ilya Metchnikoff working at Pasteur Institute suggested that longevity originates from a use of lactic acid bacteria found in sour dairy products.[1,2] The term probiotic was introduced by Lilly and Stillwell four decades ago. They defined probiotics as any organism or substance, which contributes to the intestinal microbial balance in animals.[3] Nowadays, the main and mounting interest in probiotics is related to human health, as indicated by approximately 300 peer-reviewed articles and 60 literature reviews about the topic during the year 2005 alone. The health benefits of specific probiotics have been demonstrated in several studies and a number of authors have applied the meta-analysis method for assessing such studies providing further information on efficacy.[4-6] This review will highlight current developments concerning the selection of probiotics, the role of probiotic viability for their action and their potential use both in infant's nutrition and treatment and prevention of allergic diseases. We will also discuss recent findings concerning molecular mechanisms of probiotic action both from the host's and the bacterium's perspective. In addition, new areas of probiotic research, genetically constructed strains and nutridynamics, are briefly covered.

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Definition of a Probiotic

Several definitions for probiotics have been proposed. In Europe, a probiotic has been defined by the ILSI (International Life Sciences Institute) Europe working group as "a viable microbial food supplement which beneficially influences the health of the host".[7] This definition implies that the safety and efficacy of probiotics must be scientifically demonstrated for each strain and each product. Demonstration of health effects requires clinical intervention studies with human subjects, but also includes research on the mechanisms involved. The ILSI Europe working group defined probiotic functional foods as foods which have been satisfactorily demonstrated to beneficially affect one or more target functions in the body beyond adequate nutritional effects, in a way that is relevant to either an improved state of health and well-being and/or reduction in the risk of diseases.[8] The probiotic concept is further defined by the WHO/FAO working group (2002) setting criteria for current probiotics.[9]

Traditional Selection Criteria for Probiotics and Rationale for New Ones

The selection of probiotics has traditionally been conducted using the following criteria:

1. the origin (preferably of human origin)
2. stability to acid and bile to withstand the upper gastrointestinal conditions and
3. adherence to human intestinal mucus and mucosa
4. antimicrobial activity against pathogenic bacteria

As the current probiotic concept covers a variety of different objectives new criteria for selection of probiotics have to be addressed by selected target-specific probiotic strains. The key goals include disease risk reduction in human subjects, modification of gut function in a beneficial manner and safety. The safety issues have been addressed by the International Dairy Federation working group.[9]

Several recent developments have provided a mechanistic basis for the proposed health effects,[10] and clinically proven human intervention studies have demonstrated that a significant disease risk reduction can be achieved through the use of probiotics in specific human populations.[11-12] Thus, selection criteria should include information on the target populations' microbiota. Recent advances in DNA sequencing have made whole genomes of probiotic bacteria available and annotating the genome sequences will be important in defining the capabilities of individual probiotic strains. Hence, future selection criteria will contain genomic information, which provides necessary tools such as DNA microarrays to aid in predicting and monitoring the effects of probiotics on the expression of host genes. Incorporating microbial genomic and transcriptional information together with host gene expression data from the exposed sites may lead to the ultimate uncovering of the host-microbe interactions and thereby improve the specificity of future probiotics. We thus propose the following basis for new selection criteria (Table 1).

Table 1. Proposed criteria for selecting future probiotic bacteria for human use

1.	Clear identification of the microbiota aberrancies in the target population
2.	Identification of potential members in healthy microbiota to counteract the aberrancies
3.	Competitive exclusion studies on the model aberrancies and specific strains and their impact on associated microbiota deviations
4.	Impact on metabolic activity and diversity of the target microbiota
5.	Assess the adherence of the selected strains in several adhesion models
6.	Assess the molecular interaction of the proposed probiotic with the host within the target population
7.	Utilize genomic information to assess impact on target microbiota and target populations

Importance of Viability of Probiotics

The yogurt strains *S. thermophilus* and *L. bulgaricus* have previously been regarded as of lesser value than probiotics due to their inadequate survival in vitro acid resistance studies. However, recent studies have demonstrated that these strains are able to survive gastro-intestinal passage in vivo.[13] Accumulating evidence of the health effects of yogurt strains have highlighted the importance of viability and the probiotic potential of traditional yogurt cultures. It is now clear that also specific yogurt cultures remain viable in the human gastrointestinal tract and can also be identified from fecal samples thus indicating that some yogurt cultures may also have potential on health effects and microbiota modulation.[14]

Traditionally plate counts have been used for the enumeration of viable bacteria. However, modern methods used to assess viability of bacteria have revealed that in some cases potentially probiotic bacteria may lose their ability to grow on nutrient agar (and are thus taken 'dead' by plate counts) but are nevertheless considered viable by other enumeration methods. These approaches take advantage of alternative indicators of viability such as membrane integrity, enzyme activity, pH gradient, respiration and membrane potential.[15] By combining fluorescent staining and flow cytometry, Bunthof and Abee (2002) were able to distinguish a subpopulation of intact and metabolically active but not readily culturable cells in a population of *L. plantarum*.[16] Amor and coworkers (2002) reported sublethal injury and temporary nonculturability in bile salt-stressed bifidobacteria.[17] Lahtinen and coworkers (2005) investigated the fate of *Bifidobacterium longum* and *B. lactis* in a fermented product during storage and reported that a significant subpopulation of *B. longum* entered a 'dormant' state in which the cells were not able to grow on plates, but retained a functional cell membrane.[18] These findings indicate that the viability of stressed probiotic bacteria is a complex issue and reliable determination e.g., in fermented products may require a multi-method approach.[19] This should be taken into account in future regulations and legislation concerning probiotic products. The health effects of so-called dormant probiotic bacteria are yet to be determined and there is great demand for further research on this topic.

Probiotics Augment Gut Barrier Mechanisms

The gastrointestinal tract and the gut-associated immune system have evolved into an integrated barrier between the internal environment and the constant challenge from antigens such as food and microorganisms from the external environment.[20] The gut microbiota is an active constituent in the intestine's mucosal barrier and therapeutic strategies by probiotics for combating enteric infections as well as allergic and inflammatory conditions have been studied. *Lactobacillus rhamnosus* strain GG, ATCC 53103 (*Lactobacillus* GG) has been demonstrated to improve intestinal barrier function impaired by rotavirus infection or cow's milk antigens.[21-22] Moreover, the combination of *L. rhamnosus* 19070-2 and *L. reuteri* DSM 12246 has been shown to stabilize the impaired intestinal mucosal barrier in children with atopic eczema.[23]

Lactobacilli protected gut barrier function against brush border lesions caused by diarrheagenic *E. coli* and the colonization resistance was improved by inhibition of adherence and invasion of potential pathogens.[24-26] This inbibition of pathogens seems to at least partly be explained by stimulation of mucin secretion.[27-28] Moreover, another antimicrobial peptide, human defensin-β2, was induced by lactobacilli and *E. coli* Nissle 1917 in intestinal epithelial cells in a time and dose-dependent manner.[29] VSL#3, a probiotic compound of 4 strains of lactobacilli, 3 strains of bifidobacteria and *Streptococcus thermophilus*, has also been shown to up-regulate production of mucins in intestinal epithelial cells in vitro via the MAPK signalling pathway.[30] The same probiotic mixture was also capable to reduce *Salmonella*-induced alterations in the cellular cytoskeleton of the intestinal epithelium by modulating the distribution of the intercellular tight-junction protein zonula occluden 1.[30]

Abundant immunoglobulin (Ig) A antibody production at mucosal surfaces contributes to the intestinal barrier function by binding and excluding harmful antigens.[31] Administration of *Lactobacillus* GG has been found to enhance IgA specific antibody-secreting cell response against rotavirus in infants suffering from the viral diarrhea.[32] The same probiotic strain increased fecal IgA

concentration in food allergic infants suffering from atopic eczema indicating the strain's potency to boost local protective IgA production in the gut mucosa.[33]

Probiotics Have Anti-Inflammatory Properties in the Gut

Certain probiotic strains have been found to elicit anti-inflammatory responses in the intestinal epithelial cells in vitro thus further strengthening the gut defence barrier. *Lactobacillus reuteri* was able to attenuate interleukin (IL)-8 secretion elicited by pathogenic *Salmonella* or tumour necrosis factor α (TNF-α) in the polarized T84 colonic epithelia model. This immunosuppressive effect was mediated by inhibition of the proinflammatory NF-κB pathway.[34] The same pathway was inhibited by *Lactobacillus casei* in *Shigella*-infected human intestinal epithelial cells.[35] Microarray DNA assay demonstrated that this effect was based on manipulation of the ubiquitin/proteosome pathway upstream of IκBα.

Lactobacillus GG has been shown to prevent cytokine-induced apoptosis in mouse and human colon cells in vitro via activation of anti-apoptotic Akt and protein kinase B and inactivation of pro-apoptotic p38 MAPK signalling cascade.[36] Recently, effects of commensal Bacteroides thetaiotaomicron and a mixture of probiotics (*Streptococcus thermophilus* and *Lactobacillus acidophilus*) on TNF-α- and interferon-γ-induced dysfunction in human intestinal epithelial cells were compared.[37] Both the probiotic cocktail and the commensal restored normal ion transport and reversed a decrease in the transepithelial resistance and an increase in the epithelial permeability induced by the cytokines. Of note, signal transduction was even more widely affected in the probiotic-treated epithelial cells than the commensal-treated cells.[37] These findings indicate that probiotics manipulate different intracellular signalling pathways of the intestinal epithelium to maintain local equilibrium and thus strengthen the gut defense barrier.

Atopic Disease is a Target for Probiotic Intervention

Allergic diseases, such as atopic eczema, allergic rhinitis and asthma, have reached epidemic proportions in the developed and also in many developing countries.[38] T helper (Th) 2-skewed immune response is a hallmark of allergic immune response and atopic disease in the gut and other organs of allergic manifestation. Th2 cells produce several cytokines and chemokines that amplify allergic inflammation by resulting in eosinophilia and enhanced production of immunoglobulin E antibodies against ubiquitous environmental antigens.[39] By birth, however, all T-cell responses to environmental antigens are Th2-oriented.[40-41] That kind of natural immune response is a necessity for successful pregnancy.[42] As reviewed in this book by Gary Huffnagle and colleagues, appropriate postnatal microbial stimulation is a prerequisite for a new Th1/Th2 orientation, otherwise Th2-type of immune responsiveness may persist ensuing development of atopic disease. As recently reviewed, the postnatal Th2-skewed immune responsiveness may be balanced by cytokines secreted by Th1, Th3 and T regulatory cells, partially as a result of stimulation by probiotics.[43]

Atopic eczema, generally the first symptom of atopic disorders, is a pruritic chronically relapsing inflammatory skin disease that often manifest during early childhood. The complex pathophysiology of the disease appears to result from an interplay between susceptibility genes, impaired barrier functions of the skin and the gut, aberrant gut microbiota, immunological dysregulation, together with bacterial and viral infections and other environmental factors.[44-46] Aberrant barrier functions of the skin epithelium and gut mucosa leads to greater antigen transfer across the mucosal barrier and the routes of transport are altered, thereby evoking aberrant immune responses and release of proinflammatory cytokines with further impairment of the barrier functions. Such increased inflammation would lead to further increases in intestinal permeability and in a vicious circle of increasing allergenic response and sensitization, dysregulation of the immune response to ubiquitous antigens in genetically susceptible individuals.

The target of probiotic therapy in atopic eczema may thus be characterized as impaired barrier functions of the skin epithelium and gut mucosa, including the role of local microbiota in these functions.

Probiotics in Clinical Studies with Allergic Diseases

To date randomized placebo-controlled trials of probiotics have mostly focused on patients with established atopic eczema and cow milk allergy. In the first randomized controlled clinical study, infants with atopic eczema and challenge-proven cow milk allergy were fed an extensively hydrolyzed whey formula with or without *Lactobacillus* GG.[47] There was a significant improvement in the clinical course of atopic eczema concomitant with a reduction in the fecal concentrations of TNF-α during the management with probiotics. Next, infants manifesting atopic eczema during exclusive breastfeeding were given probiotic-supplemented, *Bifidobacterium lactis* or *Lactobacillus* GG, extensively hydrolyzed whey formulas or the same formula without probiotics when formula feedings were necessitated.[48] In parallel with an improvement in skin condition in patients receiving probiotic-supplemented formulas, as compared to the unsupplemented group, there was a reduction in the concentration of soluble CD4 in serum and eosinophilic protein X in urine after 2 months of therapy, the total duration of the intervention being 6 months.

Subsequent studies in infants as well as older children with the condition have shown similar effects,[49-50] while others showed effects confined to children with evidence of IgE-associated allergic disease.[51] The importance of viable probiotics was demonstrated in a trial evaluating viable *versus* nonviable *Lactobacillus* GG, which was prematurely terminated due to adverse gastrointestinal effects in the group receiving the nonviable heat-inactivated strain, while alleviation of gastrointestinal symptoms has been achieved with viable probiotics.[52] Identified mechanisms in these studies include control of increased intestinal permeability and inflammatory response, with augmentation of the gut immunological barrier. More recently, moderate or severe eczema was treated with *Lactobacillus fermentum* VRI-033 PCC and a significant reduction in eczema scores was achieved with the probiotic compared to placebo.[50]

Probiotics administered pre and postnatally for 6 months to 159 children at high risk of atopic disease reduced the prevalence of atopic eczema later in infancy and childhood to half (23%) as compared with that in infants receiving placebo (46%) and the effect extended beyond infancy.[11,53] When probiotic supplementation was given to the lactating mother, the amount of TGF-β in breast milk could be promoted,[54] suggesting the anti-inflammatory cytokine network as one mechanism and breast-milk as one route of action.

Long-term colonization by probiotics or impairment of the natural diversity of the gut microbiota has been addressed with early or prenatal administration of probiotics. In a recent follow-up, probiotic administration in the first months of life was safe and well tolerated and did not significantly interfere with long-term composition or quantity of gut microbiota.[55] Moreover, the weights and lengths of the probiotic-treated children remained indistinguishable from normal.[56]

Probiotics May Have Additive Positive Effects with Infant Diet

A significant interaction has been found between probiotics and breastfeeding on the development of humoral immunity in infants.[57] The impact of probiotics and breastfeeding on the gut microecology and humoral immune responses was evaluated in a double-blind placebo-controlled follow-up study. The total numbers of IgM-, IgA- and IgG-secreting cells at 12 months were higher in the infants breastfed exclusively for at least for 3 months and supplemented with probiotics as compared with breastfed infants receiving placebo. Again, fecal *Bifidobacterium* and *Lactobacillus/Enterococcus* counts were higher in breastfed than formula-fed infants and sCD14, a soluble form of bacterial lipopolysaccharide coreceptor, in colostrum correlated with the numbers of IgM and IgA cells.[57] This finding underlines the interactions of breast-milk immunological factors, gut microbiota and diet in influencing the maturation process of gut immunity.

Probiotics have also been show to exert distinct effects on antigen transport, depending on the food matrix, e.g., the quality of protein in the diet. In a rodent model mucosal transport of degraded macromolecules has been found to be stimulated when *Lactobacillus* GG is administered together with unhydrolyzed protein, but reduced when administered with hydrolyzed protein (reviewed in 58). Such protein may thus stimulate the humoral immunity in the gut, but also affect on the induction of oral tolerance, as antigen degradation is an indispensable component in the acquisition

of mucosal tolerance. Moreover, polyunsaturated fatty acids have been shown to affect the growth and adhesion of probiotics and the protective effect of probiotics appear to evolve in joint action with the dietary intake of particular nutrients reducing the risk of allergic disease.[58]

Taken together, probiotic effects evidently act cooperatively with other nutritional compounds. Hence, no single supplement can be expected to resolve the epidemic of atopic diseases. The challenge in terms of reducing the risk of atopic eczema is to identify the mechanisms of the disease to detect specific targets for such dietary factors and their optimal combinations. Similarly, better comprehension of mechanisms of action of probiotics assists in reaching that goal.

Novel Molecular Technologies Aid in Uncovering Complex Host-Probiotic Interactions and Constructing Probiotics with New Properties

Application of new molecular technologies recently also in the field of probiotic research has yielded a substantial increase in knowledge of probiotics and their interaction with the host (reviewed in 59–60). These studies have described various mechanisms by which probiotics have adapted themselves to challenges encountered in the gastrointestinal tract. These include particular mechanisms reponsible for acid and bile resistance of certain probiotic strains. Fourteen genes and gene clusters encoding bacterial cell envelope functions were found to be up- or down-regulated upon exposure of *Lactobacillus plantarum* WCFS1 to bile acids indicating a major impact of bile acids on the integrity of bacterial cell wall.[61] Furthermore, seven of the identified genes and gene clusters encode typical stress-related functions such as those involved in oxidative and acid stress.[61] A two-component regulatory system of *Lactobacillus acidophilus* was shown to be involved both in proteolytic activity of the strain and its tolerance to acid and ethanol.[62]

In addition, data are accumulating from in vivo surveys demonstrating similar dependence of probiotic gene expression on local environmental factors in the gastrointestinal tract. Resolvase-based in vivo expression technology (R-IVET) allows identification of promoters that are induced when bacteria are exposed to different environmental conditions. A study by Bron and coworkers (2004) demonstrated that 72 *Lactobacillus plantarum* WCFS1 genes were induced during the gastrointestinal tract passage in mice.[63] Nine of these genes encode sugar transporter genes and another nine those involved in acquisition and synthesis of amino acids, nucleotides, cofactors and vitamins indicating the strain's ability to utilize local nutritional resources found in the gut. Moreover, four genes involved in stress-related functions and four genes encoding extracellular proteins were identified, reflecting the harsh conditions that *L. plantarum* encounters in the GI tract and its potential to be involved in interaction with host specific factors. It is of note that a remarkable number of the functions identified in the study have previously been found in pathogens in vivo during infection, suggesting that survival rather than virulence is the explanation for the activation of these genes during host residence. Interestingly, preliminary reports using transcriptional profiling to study in vivo gene expression of *L. plantarum* indicate that during passage through the human gastrointestinal tract the probiotic strain degrade carbohydrates by different metabolic pathways in small intestine and colon.[64] This clearly demonstrates that similar dependency of probiotic action on local gastrointestinal factors prevails also in man.

Recently, Di Caro and colleagues have used DNA microarray analysis to evaluate effects of *Lactobacillus* GG and *Bacillus clausii* on gene expression profile of small bowel mucosa in patients with endoscopically proven esophagitis.[65-66] They found that both strains altered the expression of over 400 genes, mostly those involved in immune response and inflammation, cell growth and differentiation, apoptosis, cell to cell signaling, cell adhesion and signal transduction. These studies analyzing tens of thousands of genes simultaneously clearly indicate that probiotics have much wider impact on the host's gene expression than thought before the era of microarray technologies.

Several factors contribute to effects of oral consumption of probiotics: the probiotic strain itself, its genomic structure and how the probiotic interacts with food consumed. If a probiotic is a component of the dietary product, levels of interactions are even more complex. In that case, other components of the product, the food matrix, e.g., the mold in which the food components

are embedded, stability of the preparation, etc., should all be taken into account. In addition, various host-related factors influence the outcome of probiotic use, including the host genotype and its impact on the inhabiting gut microbiota. The concept of nutridynamics has recently been introduced to describe these complex interactions of factors involved in consumption of functional foods such as probiotics.[67] By combining novel molecular methods available both in food industry and biotechnology, this holistic approach holds a promise for better understanding of complex molecular mechanisms of actions of probiotic foods.

Genetic manipulation of probiotic bacteria has created a new field of research. At the beginning of the millennium, Steidler and his coworkers constructed a genetically modified *Lactococcus lactis* that was able to secrete ample amounts of anti-inflammatory cytokine IL-10 locally.[68] This engineered bacterium was successful both in treatment and prevention of colitis in mice. In subsequent studies, they have further developed safety and viability of the product for potential future use in therapy of Crohn's disease.[69-70] In parallel, the group has also developed *Lactobacillus lactis* secreting bioactive trefoil factors that are cytoprotective and promote gastrointestinal reconstitution. Again, this invention has been shown to be therapeutic in a murine model of colitis.[71] Recently, Grangette and coworkers have demonstrated that anti-inflammatory capacity of an isogenic *Lactobacillus plantarum* mutant (Dlt$^-$ mutant) was greatly enhanced both in vitro and in vivo due to its modified teichoic acids.[72] The Dlt$^-$ mutant was deficient in D-alanylation which resulted in almost complete absence of D-alanine residues in the purified lipoteichoic acid (LTA). This change in the composition of LTA increased IL-10 production in vitro in peripheral blood mononuclear cells after exposure to the mutant strain. Remarkably, the same kind of IL-10 induction was observed also in vivo by using a murine model of colitis.[72] Similarly, Dlt$^-$ mutant was significantly more protective in the murine colitis than its wild-type counterpart. These findings imply that there are at least two different ways to boost natural anti-inflammatory effects of probiotics by genetic engineering: either by using them as delivery vehicles of anti-inflammatory agents or modifying their own immunogenic structures.

Summary

Currently, there is a substantial body of evidence indicating that probiotic bacteria have certain health benefits especially in infants. These benefits are supported by the meta-analysis of clinical interventions,[4-6] though in some cases the analysis has been conducted on probiotics in general, not with individual strains taking into account the strain specific properties. Thus focusing on bifidobacterial probiotics or *Lactobacillus* strains that promote bifidobacterial microbiota in the infant gut would be especially desirable. This property may be especially important in assisting in the development and maintenance of normal healthy microbiota during early years of life.

All probiotic properties are strain-specific and should be tested on their own in vitro and in clinical interventions. As site-specific probiotics for particular target groups are desired, the microbiota of the healthy breast fed infant may provide opportunities for the search of new strains with anti-inflammatory properties. In future, novel molecular technologies are expected to further aid in selection of potential probiotic strains for close evaluation, engineering new probiotics and understanding their exact mechanisms of action related to different clinical conditions.

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CHAPTER 6

Negative Interactions with the Microbiota: IBD

Nita H. Salzman and Charles L. Bevins*

Abstract

Mucosal surfaces are colonized by a complex microbiota that provides beneficial functions under normal physiological conditions, but is capable of contributing to chronic inflammatory disease in susceptible individuals. Of the mucosal tissues, the mammalian intestine harbors an especially high number of microbes with a remarkable diversity. Inflammatory bowel disease (IBD) is a group of chronic relapsing inflammatory disorders of the intestinal mucosa. Evidence from human studies and animal models provides compelling support that intestinal microbes play a key role in disease pathogenesis. While the existence a specific causative pathogen is possible, it appears more likely that intestinal microbes normally present as commensal microbiota may trigger inflammation and perpetuate disease in genetically susceptible individuals. There may be also a shift in the makeup of the commensal flora to a nonphysiologic composition that is more prone to disease (termed dysbiosis). Evidence supports that genetic susceptibility stems from one or more defects in mucosal immune functions, including microbe recognition, barrier function, intercellular communication and antimicrobial effector mechanisms. It is quite plausible to imagine that the chronic inflammation of IBD may in some cases be a normal immune response to an abnormal adherent invasive microbiota and in other cases an over exuberant immune response to an otherwise normal commensal microbiota.

Introduction

The complex ecosystems that colonize mammalian mucosal surfaces serve essential beneficial functions for the host, yet the parameters that define a healthy microbiota are poorly defined.[1-3] Unlike bacterial-host interactions involving defined pathogens, negative interactions between commensals and host are less clear-cut. The pathology may be caused by an abnormal microbiota (dysbiosis), immune defects in the host and in some instances a combination of both (Fig. 1). Some of the pathological conditions associated with commensals include the following: bacterial vaginosis, where evidence suggests an association with dysbiosis; erythema toxicum neonatorum, hair follicle penetration by commensals, which may likely reflect an immature immunity of newborns; necrotizing enterocolitis, a complex and catastrophic illness of premature infants, which is likely associated with an immature mucosal immune system[8] in combination with dysbiosis; cystic fibrosis, which is more clearly a host defect; celiac disease, in which the presence of dysbiosis has been noted; and finally inflammatory bowel disease (IBD), where dysbiosis and host immunity likely share a role in pathogenesis.[4-18] This chapter will focus on the latter group of negative interactions in the intestine characterizing IBD.

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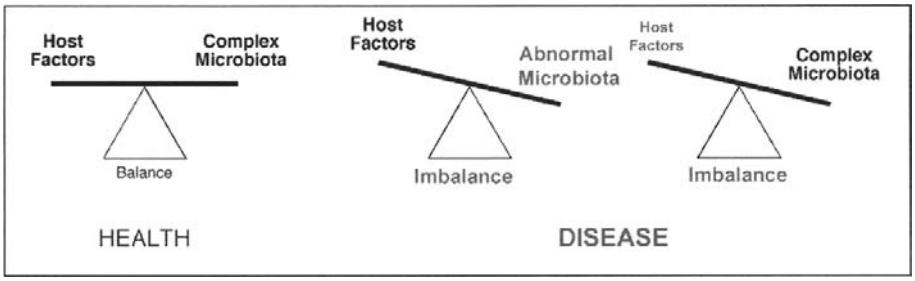

Figure 1. Simple model for balance of host-microbe interactions at mucosal surfaces. Under normal conditions, a mature fully functioning host defense system strikes balance with an abundant and complex microbiota. An imbalance leading to disease may result from either deficiencies in host factors (genetically inherited, age-related, concurrent illness, etc.), or unfavorable alteration in the composition of commensal microbiota or via virulence factors of pathogens.

IBD

IBD encompasses at least two groups of disease entities: ulcerative colitis (UC) and Crohn's disease (CD).[19] These are chronic intestinal inflammatory diseases that appear to be immune mediated, but are of largely unknown etiology.[20-22] UC is characterized by mucosal inflammation limited to the large bowel. Grossly, UC shows a region of continuous colonic involvement, often affecting the entire colon. Histologically, the acute phase is characterized by crypt abscesses and ulcerations that extend to the muscularis mucosa and is associated with a mixed inflammatory infiltrate in the lamina propria. The definitive treatment for UC involves resection of the entire large bowel and rectum, with the construction of an ileal pouch-anal anastomosis. Some patients develop significant inflammation in the ileal pouch, resulting in a secondary inflammatory disease, pouchitis. Pouchitis is associated with the clinical symptoms akin to IBD and although its etiology is unclear, pouchitis is also thought to represent an imbalance of host-microbe interactions at the intestinal mucosa, similar to IBD.[23] CD is characterized grossly by discontinuous mucosal involvement (skip lesions) that can occur anywhere in the GI tract and favors involvement of the terminal ileum. Often CD will involve only the ileum, both ileum and colon, or sometimes only the colon. Histologically, inflammation is noted to be transmural, ulceration and crypt abscesses are less pronounced, and granulomas are often present. Despite gross and microscopic differences, there may be considerable overlap in the presentation of IBD, often making precise categorization within this group of diseases a challenge.[24] Variations in both inherited susceptibility and clinical phenotypes suggest that neither UC nor CD is a homogeneous disorder, but rather a spectrum of diseases.

Evidence of Bacterial Involvement in Intestinal Inflammation

Animal Models

The significance of intestinal bacteria in inciting and perpetuating colitis has been demonstrated in a variety of murine models of IBD. Spontaneous development of colitis has been seen in a variety of rodent models, including the IL-10 knock out (K/O) mouse, IL-2 K/O mice, T-cell receptor K/O mice and HLA-B27 rats when the animals are maintained under conventional (specific pathogen free) conditions.[25-29] When these rodent strains are raised in germ-free conditions, colitis is either absent or much attenuated. Other murine colitis models, such as the SCID mouse that has been repopulated with $CD4^+CD45$ RB-hi T-cells show improvement with antibiotic treatment, also suggesting the involvement of bacteria in the development and persistence of colitis.[30]

These findings in animal models support a current hypothesis of CD pathophysiology, where an inappropriate immune response to intestinal commensal bacteria is thought to fuel mucosal

inflammation in genetically susceptible individuals. The degree to which each aspect of this triumvirate scenario (genetic susceptibility, immune reactivity, or bacteria/environmental triggers) contributes to pathogenesis and their relative contributions to perpetuation of inflammation are not yet entirely clear. One important question is whether inflammation is the result of an abnormally aggressive immune response to a normal commensal microbiota, or a normal immune response to an abnormal microbiota or pathogen.

Human Disease

There is a long history of clinical observations that also support the importance intestinal bacteria in the pathogenesis of IBD.[31] For example, diversion of the fecal stream is effective in ameliorating CD.[32,33] Although not a mainstay of therapy, antibiotics seem to provide a benefit both in acute flares and maintenance of remission of CD.[34-36] In some cases, the broad-spectrum antibiotics metronidazole and ciprofloxacin appear to be as effective in disease exacerbations as more conventional therapies.[34,37,38] In addition, the luminal contents of the small bowel have been shown to trigger inflammation.[39] There are reports of an increase in mucosal associated bacteria in the neo-terminal ileum after ileocecal resection for CD and this increase may be associated with postoperative relapse.[40] And finally, T-cell responses against the autologous bacterial flora have been observed in CD, where such responses were not seen in controls.[41] Together, these observations provide support that IBD may result from negative or pathological interactions between the commensal microbiota and its host.

Primary Cause—Bacteria?

Pathogenic Infection

The presence of granulomas associated with CD has influenced and supported the search for a specific pathogen as the causative agent in this pathological process. In other diseases, the discovery and identification of individual highly fastidious pathogens, such as *Tropheryma whipplei*, the causative agent in Whipple's disease, has encouraged this line of investigation.[42] Several different bacterial pathogens have been implicated in the etiology of CD, primarily *E. coli* and *Mycobacterium avium paratuberculosis* (*MAP*).

The implication of *MAP* is quite controversial, although particularly enticing, because of the granulomatous inflammation associated with this infection. In addition, bovine infection with *MAP* results in Johne's disease, a regional enteritis with similar appearance to the regional ileitis of CD.[43] The presence of environmental sources of *MAP* (contaminated meat and water, inadequately pasteurized milk) provides opportunities for this organism to infect humans. As noted, this is an area of significant contention, with many conflicting reports. Several groups have isolated *MAP* from the intestines, blood and breast milk of patients with CD.[44-47] However, several other studies have not found evidence of *MAP* in greater abundance in CD patients when compared to normal controls.[48,49] In addition to the difficulty in reproducibly detecting MAP in mucosal specimens, some of the weaknesses associated with the thought that *MAP* causes CD include the correlative nature of the supporting evidence, the absence of cell mediated immune responses to *MAP* in CD patients and the fact that the immunosuppressive therapy that is used to effectively treat CD should result in worsening mycobacterial infection. The discovery of *nod2/CARD15* genetic defects in a subpopulation of CD patients (discussed below) could support the theory of *MAP* involvement in CD. One would expect that these patients would have difficulty clearing intracellular pathogens. However, as mentioned, *MAP* has not been found in greater abundance in the ileum of these patients. It is also possible that the presence of *MAP* in the intestines of CD patients could be the result of the inflammatory process rather than the cause. Recent work has demonstrated some cross-reactivity between *MAP* antibodies and human self antigens, suggesting an etiology of autoimmunity rather than infection associated with *MAP*.[50] However, this study was correlative, not causative; and this line of work will require more mechanistic studies to advance this hypothesis.

Several other bacterial pathogens have been implicated in CD. Increased mucosal adherence of *E. coli*, as well as recovery of virulent *E. coli* from a significant number of mucosal biopsies of CD patients compared to controls, have supported a role for *E. coli* in the pathogenesis of CD.[51-53] There is also evidence that monocytes from patients with *CARD15* mutations, a genetic susceptibility to IBD discussed below, show a diminished response to *E. coli* infection, which would correlate with the hypothesized defective bacterial clearance that would be expected in these patients. One recent study of the etiology of granulomatous colitis in Boxer dogs has shown a strong association with adherent and invasive *E. coli*.[54] Again, as with *MAP*, the findings are correlative and the presence of *E. coli* could be as a result of the damaged mucosa rather that the cause.

Dysbiosis

An alternate theory has been gaining attention, supported by evidence from patients and animal models. This theory suggests that a shift in the normal balance of commensals, or dysbiosis leads to the colonization of the gut by bacteria that is more "proinflammatory" than the normal microbial ecosystem, resulting in the increased mucosal inflammation associated with IBD.[55] Early studies that used classical culture techniques to identify bacterial species noted increased numbers of gram-negative anaerobes, notably *Bacteroides* species in the feces of CD, UC and pouchitis. However, the bacterial composition of the feces is not representative of the bacterial composition of the individual sections of the GI tract, nor is it representative of the composition of the mucosal associated bacteria. In addition, classical culture methods are inadequate for the identification of a large percentage of the species found in the gut. The application of molecular techniques, using 16S rRNA sequences to enumerate and identify bacteria, have advanced our understanding of these complex ecosystems. The application of these techniques to fecal specimens from patients with CD has demonstrated high biodiversity with increases in enterobacteria.[56] In one study comparing the microbiota of patients with CD to those of normal controls, CD biopsies showed reduced bacterial diversity, with decreases in *Bacteroides*, *Eubacterium* and *Lactobacillus* species.[57] Another study also found differences, but could not distinguish between patient to patient variation and disease associated changes.[58] Work by Swidsinski et al that involved direct observation of tissue sections from CD and normal biopsies by in situ hybridization demonstrated high numbers of adherent bacteria in CD as compared to normal controls (Fig. 2).[59] The high numbers of mucosal associated bacteria were noted in both inflamed and non-inflamed mucosa, suggesting that the presence of mucosal associated bacteria was not as a result of epithelial inflammation.[59] A second study by Seksik et al comparing ulcerated and nonulcerated mucosal biopsies of CD patients again revealed high biodiversity and no significant qualitative differences in the microbial composition between ulcerated and nonulcerated areas.[60] These findings have been confirmed and extended by a number of very recent studies.[61,62] This suggests that ulceration of the mucosa is not directly associated with dysbiosis. Together, these findings suggest that dysbiosis is more a result of an underlying mucosal host immune defect. Investigations in animal models have suggested, in some cases, that specific subsets of commensal bacteria are more effective at inducing colitis in susceptible animal models and manipulation of the microbiota of these mice results in more aggressive colitis.[63] In the IL-10 K/O model, mono-association with *E. faecalis* was more effective than *E. coli* at inducing colitis.[64,65] Other work has shown that the presence of *Helicobacter* can induce colitis in IL-10 K/O and T-cell deficient mice.[66] Taken together with the human data, it appears that an underlying host defect or combination of environmental factors may result in alterations in the microbial colonization of the host, or dysbiosis. We are still left with the question of what causes the dysbiosis and whether the host immune response is abnormally aggressive against the dysbiotic microbiota.

Primary Cause—Host?

Primary host defects, particularly those of the immune system, have been implicated as the underlying cause of IBD, as well as other negative interactions between the microbiota and the host. There is a large body of work detailing the involvement of the acquired immune system, particularly the involvement of T-cell responses in perpetuating the chronic inflammation of IBD.[20,67] Recent

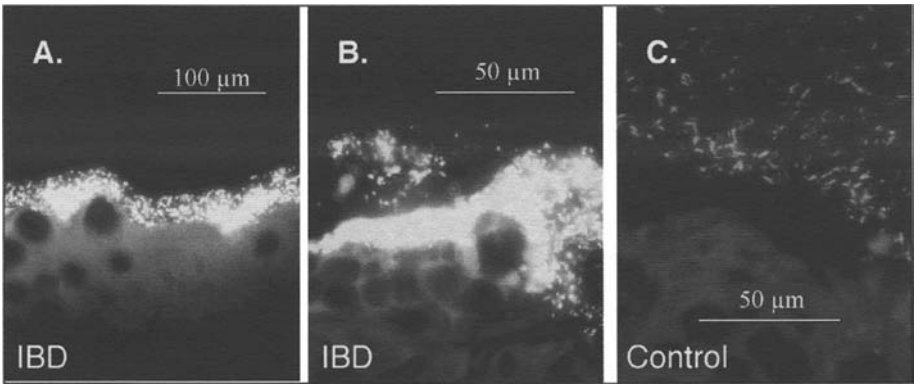

Figure 2. Mucosal adherence of bacteria in IBD. A) The ascending colon of an untreated CD patient shows a biofilm containing adherent *Bacteroides fragilis* (visualized with the Bfra-Cy3 probe). The biofilm completely covers the mucosal surface. The epithelial tissue is visualized due to autofluorescence. B & C) Triple-color FISH identifies organisms present in adherent bacteria in patients with CD (B) and irritable bowel syndrome, a non-inflammatory control (C). *Bacteroides fragilis* (Bfra-Cy3 probe) appears yellowish on a green background; the *Eubacterium rectale* group (Erec-Cy5 probe) appears dark red. Other bacteria that hybridize exclusively with the universal probe (probe Eub-FITC) appear green. An increase in adherent *Bacteroides fragilis* is detected in patients with IBD compared with controls. Data from Swidzinski, et al[59] with permission from the American Society for Microbiology Journals Department.

genetic findings have stimulated greater attention to aspects of innate immunity as a primary trigger of IBD. Mutations at several genetic loci have been associated with genetic predisposition to IBD and many of the genes identified are involved in host-bacterial interaction.

The first clear genetic association identified were loss of function mutations in *nod2* or *CARD15*, which encodes a pattern recognition receptor for muramyl dipeptide (MDP), a peptidoglycan component found in bacterial cell walls. Ligation of this intracellular receptor with MDP leads to activation of the NF-kB signaling pathway, resulting in the production of proinflammatory cytokines and subsequent bacterial clearance. The biological effects of the *nod2/CARD15* mutations associated with IBD are unclear. The receptor has been localized to monocytes and Paneth cells. Paneth cells are specialized cells that inhabit the small intestinal crypts and produce and secrete a number of antimicrobial and innate immune factors, particularly defensins.[68] Defensins are broad-spectrum cationic antimicrobial peptides. The production and secretion of epithelial antimicrobial peptides, predominantly defensins, is also important in barrier host defense.[69-71] These antimicrobials have been shown to be essential in defense against enteric pathogens and have been hypothesized to have a role in regulation of the intestinal microbiota. In animal models of targeted *nod2* knockouts, the animals show decreases in Paneth cell defensins and have increased susceptibility to enteric infection with the intracellular pathogen, *Listeria monocytogenes*.[69]

While the molecular connections between *nod2* expression and Paneth cell α-defensin expression are unclear, the results in the murine K/O system are consistent with findings in humans with ileal CD. Studies of ileal biopsies from patients with IBD have demonstrated significant reduction in gene and protein expression of human defensin 5 (HD5), the dominant α-defensin produced and secreted by Paneth cells.[72,73] Decreased HD5 expression was noted in both inflamed and non-inflamed tissue of the ileum of patients with ileal CD.[73] Patients who also had the genetic defect in *CARD15* showed even less HD5 expression than other patients.[73] Analysis of heterozygous and homozygous HD5 transgenic mice support that modest changes in Paneth cell α-defensin expression can alter microbiota (Fig. 3).[73] A current hypothesis is that reduced defensin expression and secretion could result in a change in the bacterial composition of the microbiota, dysbiosis; and allow dysbiotic bacteria closer access to the epithelium. Other support for the role of defensins

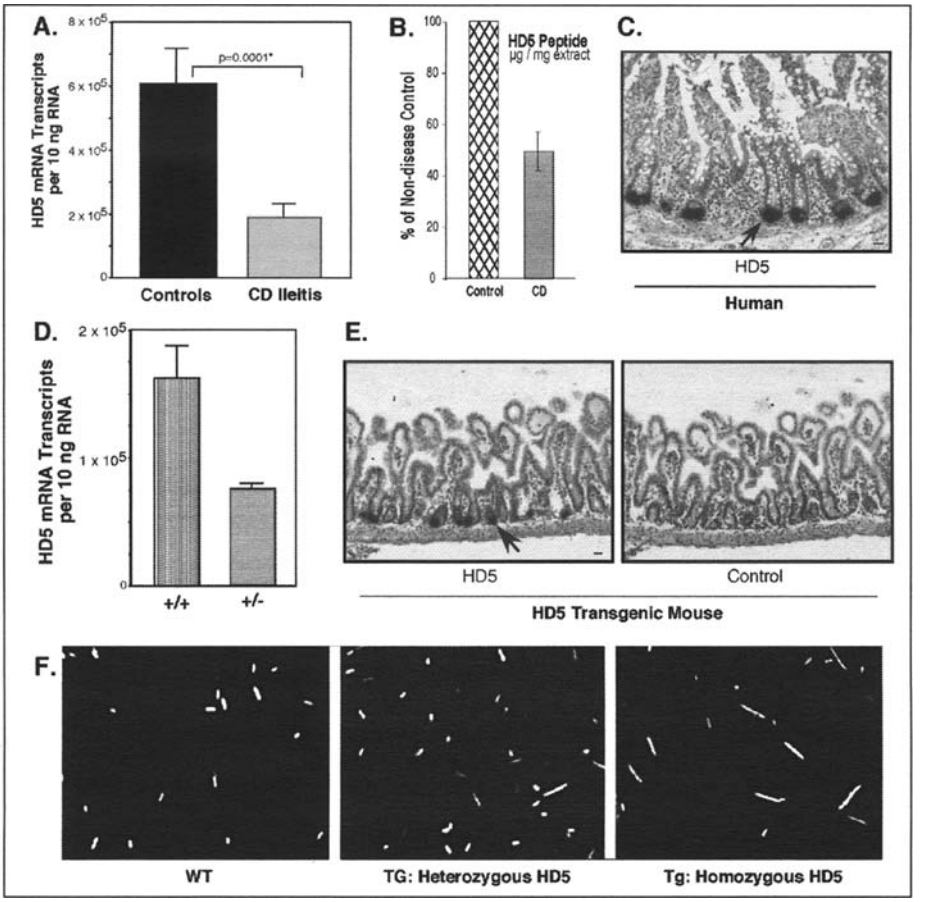

Figure 3. Reduced expression of intestinal defensins in CD of the ileum may weaken mucosal defense and lead to dysbiosis. A) Expression of HD5 mRNA in ileal specimens from controls and patients with ileal CD determined with quantitative real-time RT-PCR using external standards. Bars represent means (± standard error). B) Quantification of HD5 peptide in ileal tissue samples determined by immunoblot analysis using serial dilutions of recombinant HD5 peptide on the same gel/membrane as standard. Bars represent the percentage (± standard error) of HD5 peptide amounts in CD specimens as compared to nondisease control samples, which was set as 100%. C) Localization of HD5 mRNA in Paneth cells (PCs) of human small intestine analyzed by in situ hybridization. Arrows point to dense signal that overlie PCs. Counter-stain was H & E. Bar = 20 µm. D) Expression of HD5 mRNA in ileal specimens from heterozygous and homozygous HD5 TG mice. Data expressed as mRNA copy number per 10 ng total RNA determined with quantitative real-time RT-PCR. E) Expression and localization of HD5 mRNA in TG mouse small intestine analyzed by in situ hybridization using an antisense probe (left panel). Sense probe (right panel) was negative for hybridization signal. Expression in TG mice is similar to that seen in human ileum (Panel C). F) FISH analysis of luminal microbes in mouse ileum. Representative hybridization analysis with TR-Bac338 probe (detecting all bacteria) is shown for wildtype mice (left panel), HD5 TG heterozygote mice (middle panel) and HD5 TG homozygote mice (right panel). Morphologically, there is a graded shift in the composition of bacterial microbiota, from predominantly small bacilli and cocci in the wildtype mice (left), to a mixed population of bacilli and fusiform bacterial species in the heterozygous TG mice (middle) and finally a population of predominantly fusiform bacteria in the homozygous TG mice (right). Data from Wehkamp et al[73] with permission, © 2005 National Academy of Sciences USA.

in IBD have come from human studies of CD of the colon, where low gene copy-numbers and expression of β-defensin 2 appears to predispose to disease.[74]

A number of other associated genes appear to be involved in the maintenance of epithelial barrier integrity, including DLG5 and SLC22A4 and MDR1.[75-79] Loss of epithelial barrier integrity has been well documented in chronic intestinal inflammatory diseases. Increased intestinal permeability has been noted both in patients with CD and some unaffected first-degree relatives[80] and has been shown to be a predictor of disease relapse.[81,82] Aspects of barrier loss include the leakiness of epithelial tight junctions and the derangement of the expression and distribution of claudins, among the critical components comprising the tight junction.[83] The alterations in claudins were only seen in active IBD, not in quiescent disease. This suggests that this aspect of barrier loss is caused by the inflammation rather than an inciting cause. Nevertheless, leakiness of the intestinal barrier could allow bacteria and antigens from the intestinal lumen access to the host immune system.

Another aspect of barrier function is the maintenance of the protective mucus coating of the intestinal epithelium. Targeted deletion of *muc2*, the product of which is a component of colonic mucus, renders mice more susceptible to dextran sodium sulfate (DSS) induced colitis.[84] This reiterates the importance of each portion of the protective mucosal barrier.

Most recently, *IL23R*, which encodes a subunit of the IL-23 cytokine receptor, has been identified as an IBD susceptibility gene.[85] The involvement of this gene in IBD appears complex. Recent work in murine models has shown a requirement for IL-23 in T-cell mediated infectious and immune colitis.[86,87] IL-23 is a cytokine that is primarily secreted by monocytes, macrophages and dendritic cells and has been shown to drive the development of TH17 $CD4^+$ T-cells.[88-90] These T-cells produce IL-17, IL-6 and TNF-α, all associated with chronic tissue inflammation.[91] IL-23 also induces similar inflammatory cytokine production by monocytes and macrophages.[92] Although IL-23 is among the most recent addition, the involvement of chemokines and cytokines in mucosal inflammation and negative bacterial-host interactions has been carefully examined, both in humans and in animal models (for review see ref. 93). Since one of the hallmarks of inflammation in IBD is the recruitment of neutrophils, the involvement of increased IL-1 and IL-8 have been noted, as well as IL-12. The targeted deletions of IL-2 and IL-10 have already shown the significance of these cytokines in mucosal inflammation as the result of host-bacterial interaction.

One of the dominant cells producing inflammatory cytokines, the dendritic cell (DC), may play a central role in mediating the inflammation associated with host-bacterial interaction+. This is supported by evidence from the SCID mouse colitis model, in which increased numbers of DCs are found in the colons of affected mice and produced a strong proinflammatory cytokine response in lamina propria T-cells.[94]

DC's are integral to directing the type of mucosal response generated by the host. Lamina propria DCs have been shown to function by extending their processes through the intestinal epithelium to sample luminal bacterial contents.[95] Very exciting and recent work by Chieppa et al has shown that this antigen sampling can be triggered by intestinal epithelial cell TLR signaling in the small intestine.[96] As monocytes differentiate into DCs, they generate distinct cytokine profiles to different commensal bacteria as well as pathogens.[97-99] This implies that DCs drive the inflammatory profile of mucosa-associated T-cells depending on the type of bacteria samples and suggests a mechanism by which dysbiosis triggers and perpetuates chronic inflammation.

Concluding Comments

Although there are many clear benefits for the metazoan host to maintain and foster a complex endogenous microbiota at mucosal surfaces, under some conditions these colonizing microbes can assume a pathogenic role. In individuals who inherit one or more susceptibility genes for IBD, the intestinal microbiota may fuel the onset and perpetuation of mucosal inflammation. With an intact mucosal immune system and healthy microbiota, a balanced ecosystem will exist at the intestinal interface, marked by what may be regarded as a controlled state of physiological inflammation (Fig. 4, left). This balance may be perturbed if the host inherits a weakened mucosal immune system (Fig. 4, center). The best-characterized example of such an inherited susceptibility is a mutation

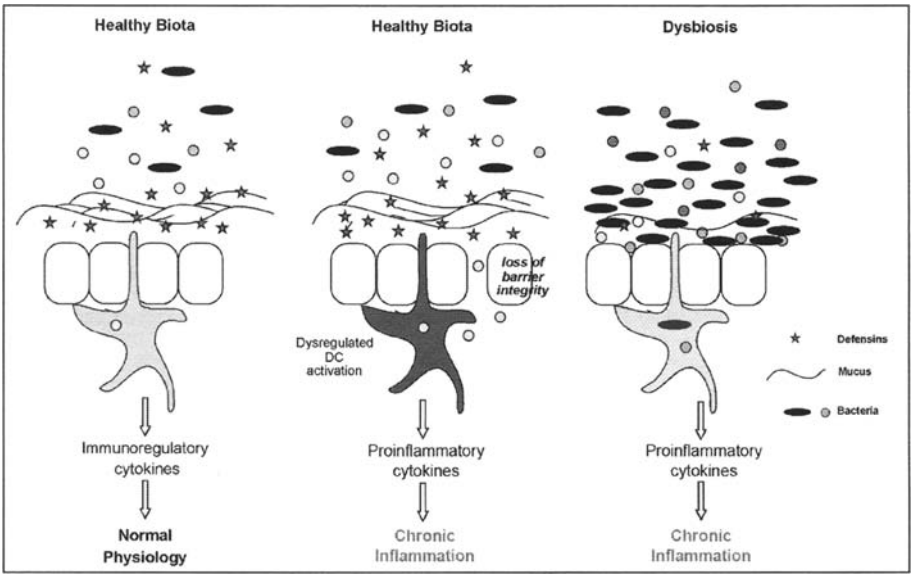

Figure 4. Proposed model for the interplay of intestinal bacteria and mucosal immune defenses in health and disease pathogenesis of IBD. The healthy intestinal tract is characterized by a delicate balance between host mucosal defenses and intestinal microbes (left). This balance may be disturbed by defects in mucosal immune functions, including microbe recognition, barrier function, intercellular communication and antimicrobial effector mechanisms (middle). An unfavorable alteration in the composition of the microbiota, dysbiosis, may fuel chronic mucosal inflammation (right). Reduced expression of intestinal defensins may be one mechanism causing an alteration in the microbiota. With further progression of disease bacterial adherence and mucosal invasion could provoke the inflammatory response.

in *nod2/CARD15*, with defective microbial recognition and cellular function. Other possibilities may include defects in barrier function, faulty intercellular communication and ineffectual immune effector mechanisms. In some cases, weakened defenses may directly alter the microbiota; such has been proposed for ileal CD where reduced Paneth cell α-defensin expression is thought to shift the microbiota unfavorably. In other cases, an altered microbiota may be the result of prolonged inflammation itself. Regardless of its etiopathogenesis, dysbiosis, the alteration of the normal commensal microbiota to a less beneficial composition, is embodied in leading hypotheses on the perpetuation of the chronic inflammation of IBD (Fig. 4, right). Defects in any of multiple genes key to maintaining a healthy, balanced ecosystem in the intestine could, in principle, manifest similarly as chronic mucosal inflammation with dysbiosis. Multiple mechanisms converging on a similar disease phenotype is consistent with both animal models of IBD and likely the human disease as well. Irrespective, of the precise etiology, further focus on host-microbe interplay may identify both insights on mechanisms of disease and new therapeutic strategies for IBD.

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CHAPTER 7

Diet, Immunity and Functional Foods

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Abstract

Functional foods (specific nutrient and/or food components) should beneficially affect one or more target functions in the body. The use of functional foods as a form of preventive medicine has been the subject of much research over the last two decades. It is well known that nutrition plays a vital role in chronic diseases, but it is only recently that data relating to the effects of specific nutrients or foods on the immune system have become available. This chapter aims to summarize the effects of some functional foods (e.g., prebiotics and micronutrients) on the immune system. It should be noted, however, that studies into the role of functional foods with regard to the human immune system are still in their infancy and a great deal of controversy surrounds the health claims attributed to some functional foods. Consequently, thorough studies are required in human and animal systems if we are to move towards developing a functional diet that provides maximal health benefits.

Introduction

Discoveries in the biosciences in recent years have provided evidence that, beyond nutrition, diet may also modulate various bodily (including immune) functions that are relevant to the host's health. These discoveries are shifting nutritional concepts from identifying a 'balanced' diet (ensuring an adequate intake of nutrients while avoiding excessive intake of those nutrients that can contribute to disease, e.g., fat and salt) to an 'optimized' nutrition. The outcome of 'optimized' nutrition is to maximize life expectancy and quality by identifying food ingredients that are able to improve the capacity to resist disease and enhance health when part of a 'balanced' diet and lifestyle. The latter provides a concept of functional foods, which was initiated in Japan in the late 1980s as a marketing term (linking medical and food sciences).

Functional foods can not be categorized with a single definition due to their novelty and diversity.[1] Although the scientific working definition of functional foods varies across geographical regions, all are in agreement that functional foods (specific nutrient and/or food components) should beneficially affect one or more target functions in the body. Thus, a general definition for functional foods states that 'a food can be regarded as functional if it is satisfactorily demonstrated to affect beneficially one or more target functions in the body, beyond adequate nutritional effects, in a way that is relevant to either improved stage of health and well-being and/or reduction of risk of disease'.[2] Quantitative evaluation of modulation of these target functions is required to scientifically substantiate the claims attributed to a particular functional food; this can be done by measuring changes in the serum or other body fluid concentration of a specific metabolite, protein or hormone, a change in physiological parameters (e.g., blood pressure or gastrointestinal transit time) and/or a change in physical or intellectual performance.[1]

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Functional foods have two main uses in terms of their action upon the immune system: (i) to overcome/prevent the effects of undernutrition and (ii) as aids in the treatment of chronic clinical conditions. Undernutrition impairs the immune system and suppresses immune functions that are essential to host protection. This state can be the result of insufficient intake of energy and macronutrients and/or deficiencies in specific micronutrients.[3] The component that makes a food 'functional' can be either an essential macronutrient with a specific physiological effect (e.g., omega-3 fatty acids) or an essential micronutrient if its intake is above the daily recommendations. Furthermore, it can be a non-essential food component (e.g., prebiotics) or a food component without nutritive value (e.g., probiotics or phytochemicals). While the beneficial effect of essential nutrients on the functioning of the immune system has been well documented, the effect on the immune function of non-essential and nonnutritive food components is a relatively recent subject of interest and thus is less well documented.[4] It is clear from studies conducted in animals, and the limited number conducted in humans, that there are a number of nutrients whose availability at an appropriate level is essential if the immune system is to operate efficiently.[3] It is also clear from recent studies that the introduction of functional foods into the diets of patients with clinical conditions can help ameliorate disease symptoms.[5,6]

The following text describes those functional foods for which data are available in relation to their effects on the immune system. Special attention is paid to the so-called 'colonic functional foods', which have been shown to affect the gut's immune response. Details are also given for some emerging functional foods that may or may not have immunomodulatory properties.

Colonic Functional Foods

The human body is host to a large number of commensal bacteria, with most residing in the gut. The large intestine is, by far, the most densely populated area of the gut and its resident microbiota plays a key role in nutrition and health as well as the proper functioning of the immune system.[7] The composition of the microbiota is influenced by various environmental and genetic factors, with dietary residues considered the most important of these. Dietary substrates reaching the large intestine are able to influence the number of bacteria (in terms of total and specific populations) present and metabolic byproducts from bacteria utilizing these dietary substrates can affect the gut-associated lymphoid tissue (GALT)—the largest component of the immune system. Consequently, dietary modulation of the intestinal microbiota is the main purpose of many current functional foods. This modulation of the intestinal microbiota by dietary means is also the basis for the pro, pre and synbiotic concepts, all of which rely upon enhancing the beneficial components of the intestinal microbiota, namely the bifidobacteria and lactobacilli. While the probiotic concept relies upon the use of live microbial supplements to modulate the microbiota, the prebiotic concept relies upon the use of nondigestible food ingredients that selectively stimulate the growth and/or activity of beneficial groups of bacteria indigenous to the colon.[8,9] The synbiotic concept is a combination of the pre and probiotic concepts.

The first records of ingestion of live bacteria by humans are over 2000 years old, but it was not until the beginning of the last century that probiotics were given a scientific basis through the work of Metchnikoff.[10,11] He hypothesized that the normal gut microbiota could exert adverse effects on the host and that consumption of 'soured milks' reversed these. Since these early observations, attempts have been made, especially in the last 20 years, to modulate the gut microbiota through the use of probiotics and these remain the most tried and tested modulators of the intestinal microbiota; their use and action have been described elsewhere in this book, so they will not be discussed further in this chapter.

Prebiotics

Any dietary material that enters the large intestine is a candidate prebiotic. This includes carbohydrates such as resistant starch and dietary fiber as well as proteins and lipids. However, current prebiotics are confined to nondigestible oligosaccharides (NDOs). These escape enzymatic digestion in the upper gut, enter the cecum without change to their structure and confer the degree of

selective fermentability that is required. Their complete fermentation by the colonic microbiota, resulting in the production of short-chain fatty acids (SCFAs), lactic acid and energy, is indicated by the fact that NDOs are not excreted in the feces.[12]

Oligosaccharides are sugars consisting of between 2 and 20 saccharide units. Some occur naturally in breast milk and certain foods such as leek, asparagus, garlic, onion, chicory, wheat, oat and soybean. However, these naturally occurring oligosaccharides can not exert a prebiotic effect in their native state, due to their low concentrations, so are produced commercially through the hydrolysis of polysaccharides (e.g., dietary fibers and starch) or through catabolic enzymatic reactions from lower molecular weight sugars. Currently, there are over 20 different types of NDOs on the world market: the most commonly used and cited prebiotics are listed in Table 1. Of these, inulin, fructooligosaccharides (FOS), transgalactooligosaccharides (TOS) and lactulose have been the most thoroughly investigated and for these a prebiotic effect has been proven.[13]

Effects of Prebiotics on Immunity

The idea that prebiotics could help the intestinal defense system originated from the observations that newborn babies, who have an underdeveloped intestinal host defense system, lack an appropriate capacity to defend themselves against intestinal infections. Furthermore, infants consuming their mother's milk were found to have a greatly reduced risk of diarrheal diseases and a lower risk of respiratory and other infections.[14] Human milk contains various protective components and active ingredients, including NDOs, which represent the third largest component of human milk and have been identified as the main factors involved in the development of an appropriate colonization process in infants, which in turn stimulates the maturation of intestinal host defenses.[15]

Although it is known that human milk oligosaccharides can exert a prebiotic effect, research into the immunomodulatory actions of prebiotics is very recent, with most data originating from animal models. In one study, mice were fed FOS or inulin for 6 weeks and then challenged with various tumor inducers and enteric and systemic pathogens.[16] It was found that prebiotic supplementation resulted in a significantly lower incidence of aberrant crypt foci in the distal colon as well as reduced pathogen-induced mortality. It was suggested that the enhanced immune functions were in response to changes in the composition and metabolism of the colonic microbiota. In another study, the same group investigated the effect of the same prebiotics on immune functions in mice. After 6 weeks' supplementation with FOS or inulin, increased natural killer (NK) cell activity and phagocytic activity of peritoneal macrophages were observed.[17]

In *Min* mice (a model for human colon cancer), FOS administration significantly reduced the incidence of colon tumors.[18] Furthermore, *Min* mice depleted of CD^{4+} and CD^{8+} lymphocytes developed twice as many tumors as immunocompetent mice, suggesting that the reduced incidence of colonic tumors after FOS supplementation was due to an appropriate functioning of the immune system.[19]

Increased IgA secretion and production of IFN-γ, IL-10, IL-5 and IL-6 from Peyer's patch (PP) cells and increased size of PPs in the small intestine were observed in mice after 6 weeks of FOS supplementation.[20] The IgA response and polymeric immunoglobulin receptor expression in the small intestine and the colon were observed in another study which examined the effect of FOS in infant mice.[21] Increased IgA secretion and phagocytic activity of macrophages were also observed in rats fed lactulose.[22,23] In addition, several studies with mice observed a change in PP cellularity in different regions of the gastrointestinal tract after administration of FOS.[18,20,24] These studies suggest that prebiotic fermentation in the colon can induce changes in several regions of the GALT.

Evidence for a direct effect of prebiotics on the human immune system is documented only in one trial, in which frail elderly individuals in a nursing home received 8 g of FOS daily for 3 weeks.[25] Significant increases in the total lymphocyte count and the number of CD^{4+} and CD^{8+} cells were observed, along with increased numbers of bifidobacteria. Reduced phagocytic activity of polymorphs and monocytes and the expression of IL-6 mRNA in peripheral blood monocytes were also observed and attributed to a general decrease in inflammation. However, another study showed that a nutritional supplement containing either placebo or inulin and FOS did not augment

Table 1. Prebiotics available on the world market

Name	Structure	Production Process
Inulin	α-D-Glc-(1→2)-[β-D-Fru-(1→2)-]n; n > 20	Extracted from chicory
Fructooligosaccharides	β-D-Fru-(1→2)-[β-D-Fru-(1→2)-]n; n = 1 – 9	Controlled enzymatic hydrolysis of inulin by inulinase
	α-D-Glc-(1→2)-[β-D-Fru-(1→2)-]n; n = 2 – 9	Transfructosylation of sucrose by β-fructofuranosidase
Transgalactooligosaccharides	α-D-Glc(1→4)-[β-D-Gal-(1→6)-]n; n = 2 – 5	Transgalactosylation of lactose by β-galactosidase
Lactosucrose*	β-D-Gal-(1→4)-α-D-Glc-(1→2)-β-D-Fru	Transfructosylation of lactose and sucrose by β-fructofuranosidase
Lactulose*	β-D-Gal(1→4)-β-D-Fru	Alkali isomerization of lactose
Isomaltooligosaccharides*	[α-D-Glc-(1→6)-]n; n = 2 – 5	Degradation of starch by α- and β-amylase to maltose and transglucosylation by α-glucosidase
Soyabeanoligosaccharides*	[α-D-Gal(1→6)-]n-α-D-Glc(1→2)-β-D-Fru; n = 1 – 3	Extracted from soya beans
Xylooligosaccharides*	[β-Xyl-(1→4)-]n; n = 2 – 9	Controlled enzymatic hydrolysis of xylan by endo-1, 4-β-xylanase

Abbreviations: Glc, glucose; Gal, galactose; Fru, fructose; Xyl, xylose.
*Available only in Japan.

the results of vaccination with influenzal and pneumococcal antigens in the elderly.[26] An indirect indication of improved immune status after consumption of milk fortified with *Bifidobacterium lactis* (DR-10TM) and TOS was documented in one trial where a reduced incidence of diarrhea and improved nutritional status of children were observed.[27]

It is clear from the limited number of studies done to date that more human studies with prebiotics are required to demonstrate the effect of these compounds on the immune system, especially since animal models suggest they have a beneficial effect. More studies into the effects of an altered intestinal microbiota on immune function are also needed, as this is also expected to modulate GALT activity.

Mechanisms for the Effects of Prebiotics on the Immune System

The underlying mechanisms of how prebiotics modulate the immune system are not known at present. Experimental data, however, suggest that these compounds exert effects in the GALT and also point to a few different mechanisms that might explain these effects:

- Selective changes in bacterial composition and bacterial products which modulate cytokine and antibody production;
- Production of SCFAs and their interactions with leukocytes;
- Modulated mucin production;
- Interaction with carbohydrate receptors of pathogens inhibiting their attachment to epithelial cells as well as receptors on immune cells.

Selective Changes in Bacterial Composition and Bacterial Products

It is well known that prebiotics increase the number of beneficial bacteria (i.e., bifidobacteria and lactobacilli).[28-31] Probiotics (usually bifidobacteria or lactobacilli), when administered orally, are known to increase the secretion of IgA in the small intestine and the feces and to stimulate PP B lymphocyte IgA production.[32,24] They are also known to exert effects on systemic immune functions and various immune parameters in the lungs, spleen and peritoneal cells.[35-37]

Intestinal epithelial cells are involved in both innate and adaptive immune responses and act by transducing signals from luminal pathogens to adjacent immune cells of the intestinal immune system, via specific germline-encoded pattern-recognition receptors, such as Toll-like receptors (TLRs) and cytoplasmic receptors.[38] TLRs are able to discriminate between the normal commensal biota and pathogens and induce the transcriptional activation of a number of genes mediating immune and inflammatory responses.[39] Pathogen-associated molecular patterns (PAMPs) [e.g., endotoxin (lipopolysaccharide), lipoproteins, lipopeptides and imidazoquinolines] present on diverse microbes are initially recognized by TLRs and their interaction results in the activation of intracellular signaling pathways, nuclear translocation of transcription factor NF-κB and the transcription of pro-inflammatory cytokines.[39] The changes that occur in the composition of the intestinal microbiota due to prebiotic fermentation could potentially reduce the presence of PAMPs and thereby exert a positive effect on the immune system.

Prebiotics also promote an increase in bacterial cell-wall components that are recognized by TLRs and in DNA derived from luminal bacteria that, in turn, stimulate the intestinal immune system.[40] Cytoplasmic components and cell-free extracts of probiotics have also been demonstrated to produce some of the same immune effects (e.g., IgA production by PP and macrophage stimulation) as live bacteria.[32,41]

Production of SCFAs

The major end-products of carbohydrate fermentation are SCFAs, of which acetate, propionate and butyrate are quantitatively the most important in the human colon. The production of SCFAs in the colon averages 400 mmol day^{-1}, with a range of 150-600 mmol day^{-1}.[42] All SCFAs are rapidly absorbed from the large intestine and stimulate salt and water absorption: principally, the gut epithelium, liver and muscle metabolize them, with virtually none appearing in the urine and only small amounts appearing in the feces. The three major SCFAs are trophic when infused into the colon and these trophic properties have important physiological implications in addition

to maintaining the mucosal defense barrier against invading organisms.[43] However, butyrate appears to be the most effective in this regard as it is a principal energy source for epithelial cells.[44] Furthermore, butyrate is known to suppress lymphocyte proliferation, inhibit cytokine production of Th1-lymphocytes and upregulate IL-10 production; it also suppresses expression of the transcription factor NF-κB and upregulates TLR expression.[45,46] Butyrate is also believed to protect against colon cancer as it inhibits DNA synthesis and induces cell differentiation.[47,48]

Increased SCFA production during prebiotic fermentation has been confirmed in a number of studies, although the extent to which serum SCFA levels are affected following prebiotic consumption is not known.[28,29,31] However, it has been demonstrated in a rat model that supplementing total parenteral nutrition with a SCFA mixture results in increased NK cell activity.[49] Pharmacological doses of acetate administered intravenously to both healthy individuals and cancer patients also increased NK cell activity and peripheral blood antibody production.[50] In addition, it has been shown that serum glutamine levels are raised following lactulose administration and suggested that increased SCFA levels were responsible for this (glutamine is a preferred substrate for lymphatic tissue).[51,52] Therefore, SCFA production in the large intestine could potentially reduce the requirement of epithelial cells for glutamine, making it available to the cells of the immune system.[53]

Mucin Production

The first line of defense of the mucosa against luminal contents is the mucous layer, which is mainly composed of high-molecular-weight glycoproteins (mucins) that are secreted by goblet cells.[54] The thickness of the mucous layer and the number of goblet cells varies throughout the gastrointestinal tract, and in the colon it increases distally, where the number of bacteria is also the highest.[55] In addition, mucin in the colon is more sulfated than in other regions, giving it a strong negative charge and making it less sensitive to degradation by bacterial enzymes (only about 1% of the total intestinal microbiota is able to degrade mucin).[56]

Thus far, the effect of prebiotics on mucin production has been reported in only one study, where it was shown that inulin administration resulted in increased mucin production in rats.[57] Greater mucin production was found to be associated with a lower incidence of bacterial translocation across the mucosa following dietary fiber supplementation.[58,59] Furthermore, SCFA production, especially butyrate, is known to modulate mucin synthesis, release and gene expression.[60-62] It has been shown in a perfused rat colon model that the production of acetate and butyrate from the fermentation of dietary fiber stimulates mucin secretion, but fibers do not have the same effect on their own.[63]

However, the mucous layer is a dynamic environment and there is still a lack of understanding as to what mucin-associated bacteria do and whether increased mucin production is a positive or a negative outcome. Pathogens and beneficial commensal bacteria are able to modulate mucin synthesis by regulating some of the mucin genes. Currently, there are 16 identified mucin genes, but further work is needed to fully explain the function of each of them and to identify new genes.

Carbohydrate Receptors

Studies suggest that some prebiotics are directly involved in protecting the gut from infection and inflammation by inhibiting the attachment of pathogenic bacteria or their toxins to the colonic epithelium.[64] This attachment is necessary before pathogens can colonize and cause disease and it is mediated by glycoconjugates on glycoproteins and lipids present on the microvillus membrane.[65] Certain prebiotic oligosaccharides contain structures, similar to those found on the microvillus membrane, that interfere with the bacterial receptors by binding to them and thus preventing bacterial attachment to the same sugar on microvillus glycoconjugates. For example, α-linked TOS, present in human milk, are known to have anti-adhesive properties and be capable of toxin neutralization.[66,67] Recently, a novel TOS mixture, which contains an oligosaccharide in alpha anomeric configuration, was shown to significantly decrease the attachment of enteropathogenic *Escherichia coli* (EPEC) and *Salmonella enterica* serovar Thyphimurium in vitro.[68]

In addition, immune cells also express specific carbohydrate receptors which mediate various cellular reactions when activated. For example, C-type receptors expressed on phagocytic cells, minor

subsets of T- and B-lymphocytes and NK cells as well as the dectin-1 receptor expressed on neutrophils and macrophages, are known to be activated by β-glucans from fungi, plants and yeast.[69,70] Recently, nigerooligosaccharide (an α-glucan-derived NDO) was found to stimulate NK cell activity in vitro, suggesting a direct effect of this NDO on NK cells via a specific lectin-type receptor.[71]

More studies, however, are required to determine whether these approaches will be successful for other prebiotics.

Dietary Fibers

There are many different types of dietary fiber (e.g., gum arabic, pectin, celfur, glucomannan, curdlan, guar gum and sugar beet) derived from plant material. After ingestion, these compounds pass into the large intestine intact (i.e., are neither fermented nor hydrolyzed) and are metabolized by intestinal micro-organisms. [It should be noted that dietary fibers differ from prebiotics in that they are not selectively fermented by the perceived beneficial bacteria (i.e., lactobacilli and bifidobacteria) of the large intestine.] A number of studies have shown that the fermentation of these fibers leads to changes in the function and structure of the gut and the production of gut-derived hormones.[72] Several studies have also demonstrated that dietary fibers enhance immunity. However, too few data are available to draw conclusions about the immunomodulatory properties of specific dietary fibers.

A feeding study involving adult dogs showed that adding fermentable fiber (in the form of a mixture of beet pulp, oligofructose powder and gum arabic) to the diet led to changes in the type and function of cells from different parts of the GALT. The fermentable fiber content of the diet (either 8.3 g/kg or 8.7 g/kg per day for 2 weeks) significantly altered the proportion of CD^{4+} and CD^{8+} cells and their in vitro response to mitogens.[72] Switching from the low- to the high-fiber diet led to increased mitogen responses in T-cell tissues (mesenteric lymph nodes and intraepithelial lymphocytes), but decreased responses in B-cell tissues (lamina propria and PPs): these effects were not observed when switching from the high- to the low-fiber diet. Switching to the high-fiber diet also led to increased NK cell activity. Studies in which rats were fed pectin and sugar beet, respectively, have also demonstrated an increase in CD^{4+} T-cell numbers in the mesenteric lymph nodes and in CD^{8+} cell numbers.[72] Studies in which the dietary fiber and/or its dose were changed have demonstrated a number of effects on the immune response: an increase in immunoglobulin production (mesenteric lymph node, serum and mucosal), an increase in the number of PPs, altered cytokine production in the mesenteric lymph nodes and altered leukocyte and lymphocyte numbers in the spleen, blood and intestinal mucosa.[72] Clearly, more work is needed to determine the doses and types of dietary fibers that are most beneficial to the immune system.

Other Functional Foods

Micronutrients

Numerous studies have shown that micronutrients such as zinc, selenium, iron, copper, β-carotene, vitamins A, C and E, and folic acid can influence several components of the immune system and have roles to play in disease prevention and the promotion of health.[73,74] Consequently, many of these nutrients are routinely included in, for example, breakfast cereals, juices and dairy products. The following is a brief overview of the effects that deficiencies of some of these micronutrients have on the body. Details for the beneficial effects of these nutrients are given in Table 2.

Zinc

Zinc deficiency has a marked effect on the bone marrow, decreasing the production of nucleated cells and of those that are lymphoid precursors.[3] In man, experimental or mild zinc deficiency results in decreased thymulin activity, NK cell activity, lymphocyte proliferation, IL-2, IFN-γ and TNF-α production and delayed-type hypersensitivity response and a lowered CD^{4+}-to-CD^{8+} ratio. Zinc deficiency is also associated with diseases such as sickle cell anemia and acrodermatitis enteropathica, where NK cell activity is decreased in the former and thymic atrophy, impaired leukocyte development, fewer CD^{4+} cells and reduced responsiveness and delayed-type hypersensitivity are observed

Table 2. Some functional foods and their properties. As well as giving details of the beneficial properties of the micronutrients discussed in this chapter, this table also contains details for foods/food components that are currently under investigation as potential functional foods.

Functional Food	Role(s)	Reported Effects on Immune System
Glutamine[77]	Indispensable metabolic fuel that is fully oxidized by the epithelial layer of the mucosa of the small intestine Maintains intestinal structure and function by providing precursors for anabolic pathways Supplies hepatocytes with an optimal substrate mix Provides citrulline/arginine for the whole organism	Increases numbers of mucosal macrophages and intra-epithelial lymphocytes in piglets; therefore, may be suitable in humans when pathologies develop in them linked to a loss of intestinal mucosa
Glutamate[77]	Suggested metabolic substrate for epithelial cells of the small intestine Specific precursor for the biosynthesis of glutathione, arginine and proline by the mucosa of the small intestine	No information with regard to role in gut inflammation, but strong suspicion that it plays a role in this process
Arginine[77]	Known to be conditionally essential in the small intestine of the neonate and for promoting intestinal repair Hypothesized to have a role in promoting intestinal cell migration (as a nitric oxide donator)	In a swine animal model challenged with *Escherichia coli* endotoxin, increased production of protective nitric oxide was observed in the alimentary canal (and several other organs) when the animals were given an intravenous arginine supplementation
Vitamin A[74]	Required for protection of membrane lipids from peroxidation Required for proper functioning of many different immune cells	Improved immune function has been observed in vitamin-A deficient hosts after supplementation
Vitamin C[74]	Required for protection of membrane lipids from peroxidation Plays a major and beneficial role in the prevention of cardiovascular disease, cancer and cataract	Dietary supplementation with ascorbic acid has been shown to enhance a number of lymphocyte functions, most notably in the elderly
Vitamin E[3,75]	Required for protection of membrane lipids from peroxidation Optimizes or even 'enhances' the immune system	Increased lymphocyte production, IL-2 production and delayed-type hypersensitivity in mice fed a diet supplemented with vitamin E Reduction of cardiovascular disease in humans associated with high vitamin E intake: may modulate atherogenesis through a number of mechanisms (e.g., reduction of the interaction of the endothelium with immune and inflammatory cells) Enhances cell-mediated immunity in the elderly Decreases incidence of diarrhea in malnourished infants

continued on next page

Table 2. Continued

Functional Food	Role(s)	Reported Effects on Immune System
Vitamin D[78-80]	Essential for bone health (regulation of calcium homeostasis) and immune function	Increased intake decreased prostate-specific antigen levels in men with metastatic prostrate cancer In vivo treatment of IL-10 knockout mice with active vitamin D blocked the progression of IBD and prevented their death Dietary functional form of vitamin D3 (1α,25-dihydroxyvitamin D_3) prevented onset of EAE, a multiple sclerosis-like disease whose progression is driven by T-cells, in mice
Nucleotides[77,81]	Serve as nucleic acid precursors, physiological mediators, constituents of coenzymes and sources of cellular energy via respiratory pathways Play a role in the growth, differentiation and repair of the gastrointestinal tract in infants	Enhance immunity in infants: mechanism of action is unknown, but is thought to be due to lymphoid cells requiring an exogenous supply of nucleotides for optimal metabolism and function Reduce risk of sepsis in infants
Garlic[82]	Consumption reduces the incidence of stomach, colon, mammary and cervical cancer	Preparations of garlic have been shown to stimulate natural killer cell activity and increase the proliferation of lymphocytes Has been shown to be a scavenger of free radicals (OH•)
Herbs[82]	Those rich in flavonoids, vitamin C or the carotenoids may enhance immune function	*Echinacea* promotes the activity of lymphocytes, increases phagocytosis and induces interferon production Glycyrrhizin, a major component of licorice root, induces interferon activity and augments natural killer cell activity
Mushrooms[83]	Whole mushrooms and components, in particular (1→3)-β-D-glucans, potentially exert tumor-inhibitory effects	Thought that mechanisms of immune stimulation involve T-cells and macrophages, but further work is required to confirm this
Selenium[74,84]	Involved in bone health and immune function Deficiency of selenium leads to immunocompetence	Selenium supplementation studies in man have shown increased lymphocyte proliferation in response to mitogen and increased expression of high-affinity IL-2 receptor Selenium supplementation of healthy human adults with marginal selenium deficiency improved polio virus clearance in these individuals; the same study also demonstrated increases in T-cell and CD4+ cell numbers

in the latter.[3] While zinc deficiency can affect the immune system, it should be remembered that excessive zinc intake also impairs immune responses: high zinc intakes can decrease lymphocyte and phagocyte functions and can result in copper depletion (copper also being necessary for proper immune function).

Dietary Antioxidants—Vitamins A and E

Reactive oxygen species (free radicals) are produced by phagocytes as part of the body's defense against infection. These species can cause injury to immune cells, impairing cell-cell communication and, consequently, immune responsiveness. In addition to endogenous oxidative stress, exposure to oxidants and free radicals in the environment (e.g., cigarette smoke, ultraviolet light and ozone) can contribute to the level of oxidants in the body.[75] Many anti-oxidants are obtained from the diet, but adequate amounts of neutralizing anti-oxidants are required to prevent damage to immune cells by phagocyte-produced reactive oxygen species. It has long been known that there is a link between diets rich in anti-oxidants and a reduced incidence of cancer and it is thought that this is due, at least in part, to anti-oxidants boosting the body's immune system and helping to protect it from the toxic products (i.e., reactive oxygen species) produced by the action of phagocytes.[74]

Vitamin A affects many different types of immune cell. A deficiency of this vitamin can cause defects in phagocytic activity (i.e., defective chemotaxis, adhesion and ability to generate reactive oxygen metabolites in neutrophils), impairment of T- and B-cell function and reduced NK activity, production of IFN, effectiveness of fixed fat macrophage activity and lymphocyte response to stimulation by mitogens.[74] It can also change the integrity of the intestinal epithelium, which may lead to an altered immune response that allows translocation of bacteria (i.e., the movement of intestinal bacteria to extraintestinal organs) and, possibly, systemic infection.[76]

Vitamin E is the major lipid-soluble anti-oxidant in the body and is required for protection of membrane lipids from peroxidation.[3] Vitamin E deficiency has been shown to decrease spleen lymphocyte proliferation, NK cell activity and phagocytosis by neutrophils in animals.[3] Vitamin E deficiency is also known to increase susceptibility of animals to infectious pathogens; indeed, studies in chickens, turkeys, mice, sheep, pigs and cattle have shown that an increased intake of vitamin E promotes resistance to pathogens.[3] It should be noted that the effects of vitamin E deficiency are more marked if animals are fed a diet containing a high level of polyunsaturated fatty acids. In addition, the amount of vitamin E required for maximal effect is age-dependent (i.e., increases with age due, in part, to prolonged exposure to free radicals). As noted for zinc, excessive vitamin E in the diet can impair immune functions: some studies have reported that ≥300 mg vitamin E per day can decrease the ability of neutrophils to undergo phagocytosis and to kill bacteria and decrease monocyte respiratory burst and IL-1β production.[3]

Glutamine

Glutamine is defined as a conditionally essential amino acid. Studies have demonstrated that the ability to synthesize and store glutamine may be impaired in some individuals and may affect optimal growth and renewal of cells during long-term stress, hypercatabolic and hypermetabolic states or prolonged starvation.[77] Glutamine has been shown to change the cellular structure of the piglet small intestine (particularly the ileum and jejunum) and to possibly restore the intestinal mucosa after thinning (e.g., after weaning).[77] Therefore, there may be a future role for glutamine supplementation (either via enriched foods or production by probiotic bacteria) in the treatment of inflammatory conditions in the human small intestine.[77]

Vitamin D

Very few foods naturally contain vitamin D, which is why at the turn of the 20th century more than 80% of European and American children suffered from rickets.[78] Nowadays, many foods (e.g., dairy products, orange juice, cereals and bread) are fortified with vitamin D in the US. However, only margarine and some cereals are allowed to be fortified in most European countries due to an outbreak of vitamin D poisoning in the 1940s.[78] The majority of people's vitamin D (in the form of vitamin D_3) requirement is obtained by exposure to sunlight. Circulating levels of active

vitamin D in the body mean that it can interact with tissues that have a vitamin D receptor (i.e., skin, colon, prostate, breast, heart, skeletal muscle, brain, monocytes and activated T-cells), which helps maintain cellular growth and prevents the cells from becoming malignant; therefore, there is a strong link between vitamin D deficiency and the development of cancer.[78] Deficiency of this vitamin has also been observed in patients with irritable bowel disease (IBD) and accelerates the development of experimental IBD in IL-10 knockout mice.[79]

Nucleotides

Nucleotides, like many amino acids, are considered conditionally essential. They have been added to infant formulae for many years in an effort to improve immune function. During periods of rapid growth or limited nutrient intake, or in certain disease states in which a loss of gastrointestinal mass occurs, intake of nucleotides spares the organism from de novo synthesis and may bring tissue metabolic levels to full working conditions.[77]

Summary

The examples given in this chapter demonstrate clearly that a number of foods and food components beneficially stimulate the immune system and confer health benefits upon the consumer. Although studies into functional foods and their action on the immune system are still in their infancy, it is an exciting area of research that may allow the development of foods that will obviate the need for resorting to medicines for the treatment of certain conditions. The ultimate aim of these studies would be the development of a 'functional diet' that confers maximal health benefits upon the individual.[74] However, there may be a need to develop analytical methods (biochemical and/or molecular) that allow tailoring of functional foods to a particular individual's needs. It is important, also, to consider cultural and regional aspects when developing functional diets.[74]

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CHAPTER 8

Host-Microbe Communication within the GI Tract

Christopher A. Allen and Alfredo G. Torres*

Abstract

The gastrointestinal tract is a biologically diverse and complicated system which carries out essential physiological functions that support human health, while at the same time maintaining itself as an isolated environment to prevent infection and systemic disease. To maintain homeostasis in the gut, communication between the host and residing microbial communities must occur to identify and eliminate potential pathogens which could colonize and cause damage through aggressive pro-inflammatory responses by the mucosal immune system. To prevent such events, a number of host and bacterial-mediated mechanisms are utilized to monitor the environment and initiate appropriate immune responses to invading pathogens. An essential component of this communication process between gastrointestinal microflora and the host involves distinguishing indigenous species from pathogens through ligand-receptor interactions which lead to various signaling events in host cells. Such events generally result in the development of mucosal immunity and immunological tolerance. While these signaling pathways provide a highly effective means of communication between the gut microflora and the host, pathogens have developed mechanisms to manipulate these pathways to evade detection by the immune system to persist and cause disease. These adaptations include cell surface modifications and the expression of various virulence factors in response to different immunological and hormonal components produced by the host.

The Gastrointestinal Tract

The gastrointestinal (GI) tract is the largest and most complex biological environment in humans. The GI tract can be divided into four main regions (oral cavity, stomach, small intestine and large intestine) and harbors a wide spectrum of microbial flora (up to 1,000 species) which colonizes a variety of "micro-niches" within different regions of the GI tract. Microbial loads and diversity expand distally into regions such as the gastric outlet (10^3/ml), the ileocecal valve (10^{10}/ml) and the colon (10^{12}/ml). Most of the microbial species colonizing the GI tract are classified as obligate anaerobes and live in a symbiotic state with the host. Among the predominating microbial genera within the GI tract are: *Escherichia*, *Clostridium*, *Lactobacillus*, *Bacteroides*, *Eubacterium*, *Peptococcus*, *Peptostreptococcus*, *Veillonella*, *Fusobacterium* and *Bifidobacterium*.[1] Commensal bacteria provide a number of benefits to the host, including protection from pathogenic transients, nutritional benefits and maturation of normal mucosal immunity. In return, the GI tract provides a diverse, nutrient-rich environment in which colonization is possible for both facultative and obligate anaerobes.

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Maintaining Physiological and Immunological Homeostasis in the Gut

The GI tract plays a crucial "balancing act" by both confining the microflora in the lumen, protecting the host from potential disease and maintaining the mucosal immune system in a low reactive state. To facilitate these functions, the GI tract relies on a repertoire of diverse cells and cell products. The intestinal epithelium of the gut consists of a layer of intestinal epithelial cells (IECs), supported by smooth muscle cells, which functions as a physical barrier preventing the passage of bacteria and bacterial products outside of the lumen. The tight junctions within the paracellular spaces of IECs aid in maintaining cell polarity and the strict regulation of diffusion between the lumen and lamina propria preventing the escape of enteric pathogens outside of the lumen (Fig. 1). Specialized secretory cells within the intestinal epithelia, such as the goblet and Paneth cells, produce mucins and antimicrobial-peptide defensins, respectively, which function to trap and selectively kill transient pathogens in the lumen through cytolytic activity.[2] IECs also express antimicrobial factors on their surface which can act as selective biochemical barriers to prevent the colonization of unwanted pathogens. For example, the reported surface-expressed protein, bactericidal/permeability-increasing protein (BPI), is an antibacterial/endotoxin-neutralizing molecule which can act on Gram-negative bacteria by damaging cell membranes, neutralizing lipopolysaccharide (LPS) and functions as an opsonin for phagocytosis by immune cells.[3]

The mucosal immune system participates in the maintenance of gut microbial communities by directly monitoring the luminal environment through sampling processes by specialized surface Microfold cells (M-cells) overlying lymphoid follicles and dendritic cells (DCs) residing in the lamina propria. M-cells can transport and present microbial antigens to antigen-presenting cells within lymphoid follicles while DCs extend pseudopodia-like dendrites through the tight junctions between IECs for direct sampling[4] (Fig. 1). This constant sampling process helps to maintain a state of immunological tolerance towards antigens from food and commensal bacteria, while preserving the ability to initiate both innate and adaptive immune responses upon detection of microbial pathogens. Besides sampling the lumen environment, the production and secretion of gut-associated immunoglobulins into the lumen by plasma cells within the lamina propria aids in the neutralization and clearance of pathogens and their toxins.

Host-Bacterial Interactions in the Gut

In order to maintain internal homeostasis in the GI tract and regulate immune system activity, communication among commensal flora and between flora and the gut must occur to distinguish bacterial pathogens from indigenous flora for the elicitation of appropriate immune responses. Quorum sensing (QS) is a form of cell-to-cell communication which occurs through a density-dependent recognition of signaling molecules known as autoinducers (AIs) that trigger a variety of inter- and intra-signaling pathways among bacterial communities. QS allows bacterial communities to function as a collective unit (i.e., biofilm) which provides a competitive edge to various commensal species when competing with transient pathogens for certain environmental niches such as those located in different regions within the GI tract. Such communication plays a protective role by commensal species colonizing the gut of the host (Fig. 2).[1]

In addition to QS, direct communication between bacteria and host cells within the gut plays a major role in the immunomodulation of the mucosal immune system to prevent aberrant pro-inflammatory responses toward commensal species. An important aspect of this interaction is the ability to distinguish non-pathogenic and pathogenic species. To accomplish this process, specialized surface receptors expressed by IECs known as Toll-like receptors (TLRs) detect pathogen-associated molecular patterns (PAMPs) and distinguish invading pathogens from commensal species. PAMPs include various microbial products, such as Gram-negative bacteria LPS, Gram-positive bacteria lipoteichoic acid and bacterial flagellin which are recognized by different TLR family members such as TLR4, TLR2 and TLR5, respectively. The binding of bacterial antigens to the corresponding TLRs will initiate intracellular signaling cascades, leading to transcriptional events which ultimately generate various physiological and/or immune-based responses.[5]

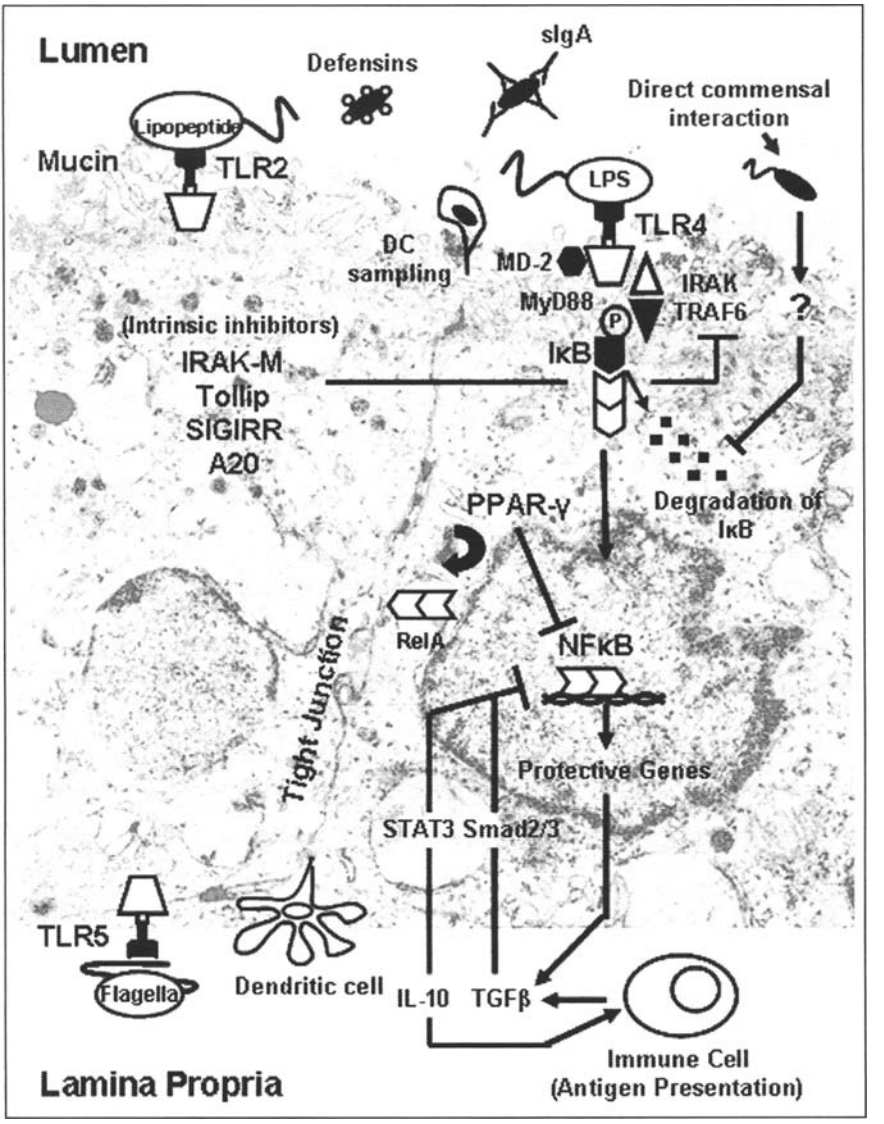

Figure 1. Host and bacterial-mediated mechanisms to maintain homeostasis in the gut. The presence in the host cell of intrinsic negative regulators of the TLR4/NFkB cascade (IRAK-M, Tollip, SIGIRR, A20 and PPARγ) helps to control the state of TLR4 activation. Furthermore, immunosuppressive cytokines (TGF-β and IL-10) induce phosphorylation signaling cascades of Smad2/3 and STAT3, respectively, decreasing NFkB activity and inflammatory processes in the gut. This complex regulatory network helps maintain host homeostasis. However, improper TLR4 signaling due to a failure of the regulatory proteins to down-regulate signal transduction leads to a constant activation and the subsequent production of inflammatory cytokines and chemokines, eventually culminating in uncontrolled inflammatory processes. In addition to this important regulatory mechanism, other signaling pathways (i.e., other TLRs), immune-mediated mechanisms (DCs, APC, sIgAs), or secretion of antimicrobial peptides (defensins), help to maintain the host homeostasis and prevent colonization by pathogenic organisms (See text for details).

Host-Mediated Regulatory Mechanisms

One of the most commonly known TLR-based pathways is the NF-κB pathway which is activated upon the binding of LPS to TLR4. Activation of this pathway leads to the translocation of the transcriptional regulator, NF-κB, from the cytoplasm into the nucleus and the transcription of genes encoding cytokines (i.e., TNF-α, IFNγ) associated with pro-inflammatory immune responses (Fig. 1). To prevent improper activation of TLR pathways, both host and bacterial-based immunomodulatory mechanisms exist to selectively regulate signaling processes. Intrinsic inhibitory molecules (i.e., Tollip [Toll-interacting protein], SIGIRR [single immunoglobulin IL-1R-related molecule], IRAK-M [IL-1R-associated kinase-M] and A20) are expressed in IECs which interrupt interactions among key signaling proteins within signaling cascades.[6] Elevated expression of SIGIRR has been shown in IECs and Tollip in IECs with attenuated responsiveness to LPS. In addition, the NF-κB inhibitor, peroxisome proliferator-activated receptor-γ (PPAR-γ), has been shown to be partially regulated by TLR4 in animal colitis models and has been suggested to play a regulatory role in intestinal inflammation.[5]

While intrinsic inhibitors exist to function in a negative feedback loop fashion, other signaling cascades can "crosstalk" with the TLR pathway to prevent immune hyper-responsiveness. Among these independent signaling cascades are the pathways induced by the immunosuppressive cytokines, IL-10 and TGF-β (Fig. 1). The NF-κB inhibitory molecules, STAT3 (IL-10 pathway) and Smad2/3 (TGF-β pathway), are activated in these pathways and help decrease NF-κB activity to maintain host homeostasis.[6] As microbial loads increase distally from the small intestine towards the colon, an enhancement in the risk of constitutive TLR stimulation by commensal species can develop. Several mechanisms exist which help alleviate this risk, which include changes in TLR expression and location (Fig. 1). In colonic epithelia, TLR4/2 expression is decreased and responsiveness is attenuated, while TLR5 expression is limited to the basolateral surface. In addition to IECs, immune cells, such as macrophages associated with the lamina propria, express low levels of TLR4/2 and are unresponsive to LPS in contrast to DCs, which maintain responsiveness to TLR3/4 ligands.[6] While TLRs play an important role in the communication between luminal bacteria and gut epithelia to regulate the immune system function, recent studies have also found a role for TLRs to prevent epithelial injury. Mice deficient in TLR4, TLR2, or MyD88 (intracellular TLR pathway component) were found to have increased mortality rates compared to wild-type mice when orally challenged with the sulfated polysaccharide dextran sulfate sodium (DSS).[7] Direct DSS exposure is toxic to the colonic epithelia of animals.

Bacterial-Mediated Regulatory Mechanisms

While host mechanisms exist to maintain homeostasis in the gut and prevent aggressive inflammatory responses triggered by residential microflora, bacterial mechanisms are also present which limit inflammation by acting through the NF-κB signaling pathway. Direct interactions between human colonic epithelia and, i.e., nonvirulent *Salmonella* strains have been shown to attenuate secretion of the pro-inflammatory chemokine IL-8. This occurs by blocking the degradation of the inhibitor IκB-α, which binds NF-κB in the cytoplasm and prevents its translocation into the nucleus to complete the pathway circuit and subsequent pro-inflammatory cytokine production.[8] The commensal anaerobe, *Bacteroides thetaiotoamicron*, has been shown to attenuate TLR signaling triggered by both flagellin and flagellated pathogens through a mechanism regulating the nucleocytoplasmic distribution of NF-κB. This mechanism involves promoting the nuclear export of the transcriptionally active NF-κB subunit, RelA, which is dependent on interaction with the NF-κB-associated nuclear protein PPAR-γ (Fig. 1).[9]

The Role of Gut Flora in Immune System Development and Immunological Tolerance

As previously mentioned, commensal flora play a crucial role in both immune system development and immunological tolerance. Studies using germ-free animals have shown that immune system development is greatly hindered in the absence of normal commensal microflora. These

changes include the underdevelopment of lymphatic tissues and components, delayed migration of antibody-producing cells in response to bacterial antigens, reduced antibody diversity and lowered lymphocyte responsiveness. Intestinal colonization with defined commensal bacteria or stimulation of TLRs (i.e., TLR2) enhances overall immune system development and function.[10] Among these developmental changes were an increase in lymphocyte infiltration of the gut mucosa, germinal center development within Peyer's patches and production and differentiation of antibodies. Reconstitution of microflora has also been found to correct several immunologic defects found in the absence of a bacterial microflora. Administration of bacterial polysaccharide from the symbiotic bacterium *Bacteroides fragilis* was shown to correct immune defects in germ-free animals.[11] In addition to immune system maturation, the presence of microflora impacts normal intestinal structure and physiology. Stimulation of TLR2 on colonic epithelial cells with bacterial antigens (i.e., lipopeptide and peptidoglycan) has been shown to initiate tight junction development, including apical tightening/sealing and increased transepithelial electrical resistance.[12] In the absence of commensal colonization, increased mucus accumulation, water retention, extended epithelial cell cycles and decreased peristalsis was found to occur in the large intestine of germ-free models when compared with control animals.[2]

Immunological tolerance in the gut cannot be achieved without the assistance of commensal interaction. Immune responses to normal flora allow the development of regulatory T-cells and the production and secretion of IgA (sIgA), both of which function as important tolerance mechanisms. Regulatory T-cells produce immunosuppressive cytokines, such as IL-10, which helps attenuate pro-inflammatory responses that can otherwise potentially contribute to inflammatory bowel diseases. DCs play an important role in T-cell recruitment into the lumen and in regulatory T-cell development.[5] TLR signaling in response to commensal antigens (i.e., LPS) enhances the surface expression of chemokine receptor CCR9 and integrin $\alpha_4\beta_7$ for the generation of gut-tropic T-cells.[13] Increased expression of these surface molecules also promotes T-cell migration into the gut through interactions with gut endothelia expressing mucosal addressin cellular adhesion molecule-1 (MAdCAM-1) and intestinal epithelia expressing thymus-expressed chemokine ligand (TECK).[14,15] Another mechanism through which DCs contribute to the development of immunological tolerance is the selective induction of sIgAs. DCs containing commensal bacteria have been shown to be used as a priming tool for the selective induction of sIgAs to prevent mucosal penetration by commensal bacteria, thus initiating systemic pro-inflammatory responses in the gut.[16] Commensal-mediated TLR signaling has been shown to play an essential regulatory role based on in vivo studies. Stimulation of TLR9 with bacterial DNA has been shown to reduce inflammation in animal colitis models, while TLR4 signaling by intestinal flora has been shown to elicit protective effects in animal models for food allergies.[17,18]

Commensal Bacteria, Mucosal Immunity and Development of Inflammatory Disease

Commensal microflora, at an optimal composition, prevent attachment and multiplication of pathogenic bacteria on luminal surfaces and their invasion and spread into gut epithelia.[10] Intestinal microflora plays an important role in resistance to infection both by direct interaction with pathogenic bacteria and its influence on the function and activity of the immune system. Components of intestinal microflora play a crucial role in the postnatal development of the immune system. During the early postnatal period, the intestinal microflora stimulates the development of both local and systemic immunity. Later on, these components evoke, on the contrary, regulatory (inhibitory) mechanisms intended to keep both mucosal and systemic immunity in balance.[2]

In the intestines, commensal bacteria that have the ability to penetrate mucins and resist host antimicrobial peptides, can potentially reach close proximity to epithelial cells that line the intestine. In this scenario, TLRs and nucleotide-binding oligomerization domain isoforms (NODs) play a crucial role in their recognition by the mucosal immune system. Upon the recognition of specific microbial components, TLRs trigger both innate and adaptive immune responses that eliminate pathogens and shape the intestinal microflora. Among these innate immune responses is

the synthesis of antimicrobial peptides, pro-inflammatory cytokines and chemokines and secondary anti-inflammatory responses required for the resolution of inflammation.[19,20] TLR signaling also impacts subsequent T-cell responses through activation of DCs.[21] The NOD proteins, which localize to the host cell cytosol also trigger both innate and acquired immune responses following cellular uptake and recognition of muramyl dipeptide and meso-diaminopimelic acid motifs, components of Gram-negative and Gram-positive organisms.[22]

The ability of epithelial cells to mount rapid innate immune responses to luminal pathogens has thus far been explained by rapid translocation and/or internalization of pathogen-specific ligands and migration to cognate TLRs.[10] It is well known that viable microorganisms, as well as the release or secretion of bacterial components, are responsible for several of these immunomodulatory effects. Some of the released bacterial components which produce strong effects on the innate and/or adaptive immunity include LPS, peptidoglycan, CpG-DNA motifs, heat shock proteins and superantigens.[10] For example, LPS (endotoxin), a major outer surface component present in all Gram-negative organisms, acts as a strong stimulator of the innate immune system. Within minutes of recognizing LPS, an array of cell types expressing relevant pattern recognition receptors (PRRs; CD14 and TLR4) initiate defensive actions that mediate protection against microbial pathogens, including the production of reactive oxygen intermediates and secretion of inflammatory cytokines. Cytokines initiate a cascade of signals to cells of the adaptive immune response, preparing them for the development of antigen-specific immune responses. LPS exposure also results in production of defensins, which comprise several distinct families of antibacterial, antifungal and antiviral peptides.[23]

Another bacterial component that can stimulate the immune system and initiate an inflammatory response is the flagella found on the surface of several commensal and pathogenic bacteria.[24] Interestingly, the predominance of flagellated commensal bacteria in the human gut is several orders of magnitude higher than that encountered in a typical infection by flagellated pathogens. The question therefore arises as to how the gut accommodates such high levels of flagellin in the absence of an inflammatory response because monomeric flagellin, through its activation of TLR5 and NF-κB, is a potent pro-inflammatory ligand.[24] One possibility is that pro-inflammatory responses induced by commensal bacteria are rapidly attenuated, either by host systems or by gut bacteria, in ways analogous to the immune evasion strategies used by pathogenic bacteria. However, the ability of certain commensal bacteria to attenuate NF-κB has been recently demonstrated.[10]

Once the bacterial component has reached the specific TLR, the signaling is carefully regulated in the healthy gut. For example, TLR2 induces both pro-inflammatory and anti-inflammatory cytokines.[20] Other host mechanisms exist that modulate TLR-mediated responses, including IL-10, transforming growth factor β (TGF-β), SIGIRR, anti-inflammatory TLR9 signaling, cytosolic NOD2, etc., (Fig. 2).[10] The genetic defects in these regulatory systems can predispose subjects to inflammatory diseases. Specifically, mutations in the *NOD2* or caspase activation recruitment domain 15 (*CARD15*) genes have been linked to an enhanced susceptibility to Crohn's disease. Although wild-type NOD2 is an activator of NF-κB, intact NOD2 signaling appears to inhibit the TLR2-driven activation of NF-κB.[25] NOD2 deficiency and the Crohn's disease-like Card15 mutation are associated with aggressive Th1 responses that can promote tissue damage and inflammatory diseases.[25] These Th1 responses might be directed towards commensal bacterial flagellins.[24] Local immune responses directed against bacterial flagellin might exacerbate the disruption to the natural balance of bacterial groups associated with inflammatory bowel disease.[10]

Novel Mechanisms for Host-Pathogen Crosstalk within the GI Tract

The human host restricts the growth of invading bacteria by both innate and adaptive immunity. As indicated above, one important component of innate immunity is the production of cationic antimicrobial peptides. Bacteria have evolved mechanisms to resist killing by antimicrobial peptides. These mechanisms have been best characterized for *Salmonella enterica* serovar Typhimurium but are also present in other bacterial pathogens.[26] Resistance to antimicrobial peptides is typically acquired by modifications of the bacterial cell surface, for example, the lipid A portion of the LPS

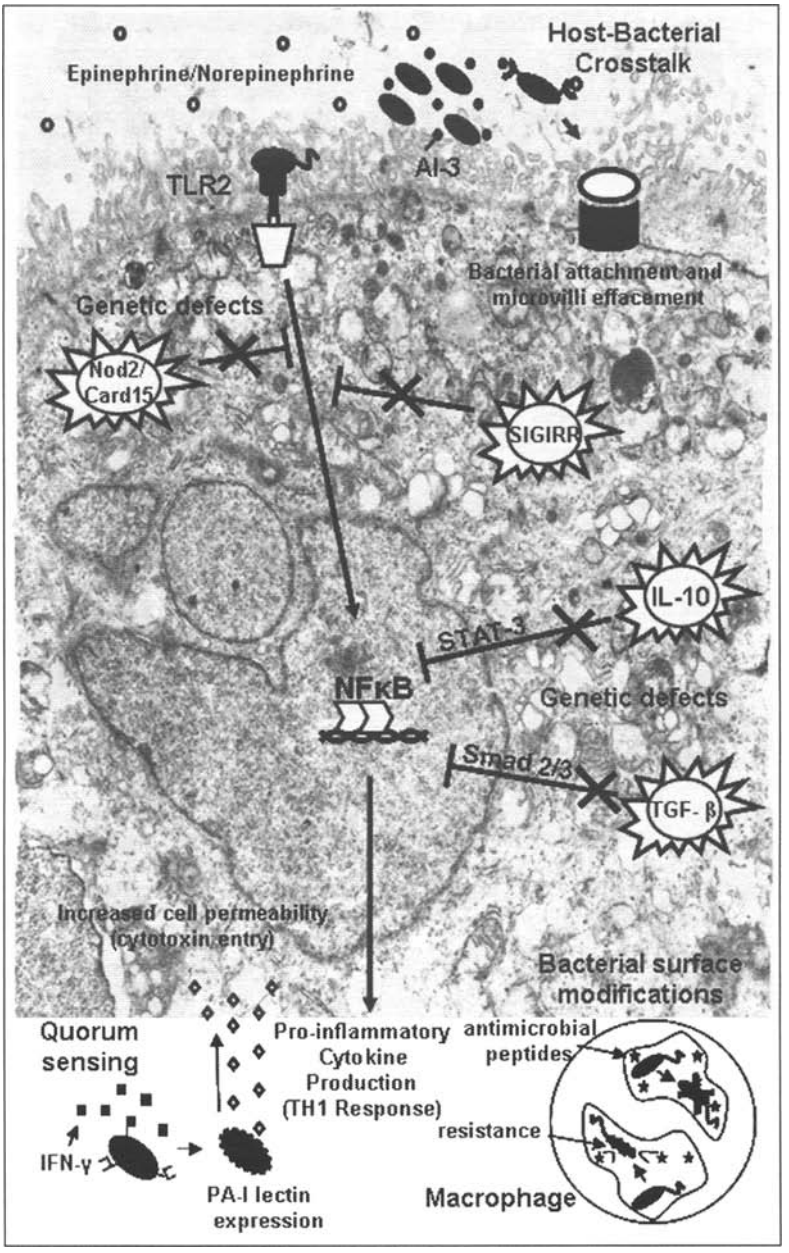

Figure 2. Novel mechanisms for host-pathogen crosstalk within the gut and development of inflammatory disease. Several host mechanisms modulate TLR-mediated responses (i.e., IL-10, TGF-β, SIGIRR, NOD2, CARD15, etc.,) and help in maintaining a state of controlled inflammation. Genetic defects in these regulatory mechanisms are associated with adaptive immune (Th1) responses that can promote tissue damage and inflammatory diseases. In addition, pathogens have found ways to evade both innate and adaptive immunity, i.e., pathogenic bacteria can resist the effect of antimicrobial peptides by modification of their cell surface or by inducing expression of virulence factors which are dependent on the quorum sensing mechanisms.

(Fig. 2). Lipid A modifications, induced during *Salmonella* invasion of macrophages, play a role during bacterial virulence and, when modifying a variety of Gram-negative bacteria, are regulated by a two-component system termed PhoPQ.[26,27] The *Salmonella* PhoPQ system is activated in vivo within acidified macrophage phagosomes and repressed in vitro during bacterial growth in high concentrations of divalent cations. Furthermore, the PhoPQ system is active at low cation concentrations in vitro and fully induced during bacterial replication in macrophages.[27] Because PhoPQ is essential to protect bacteria from antimicrobial peptides, the question arises as to whether these peptides could serve as signals for PhoQ activation. Recent work by Bader et al deciphered a mechanism by which the PhoQ sensor kinase of *Salmonella* is switched on by host cationic antimicrobial peptides, leading to changes in gene expression that enable *Salmonella* to combat the host immune response.[28] This is the first study revealing a novel example of a sensor mechanism from a bacterial pathogen that is activated by innate immune effectors.

Another example of mechanisms used by pathogens exploiting changes in the host immune system occurs during *Pseudomonas* infections. Like other opportunistic pathogens, *Pseudomonas aeruginosa* can cause lethal infections by invading a host that is both physiologically stressed and immunologically compromised. It has been demonstrated that within the intestinal tract of a stressed host, the lethality of *P. aeruginosa* is dependent on the expression of the adhesin known as PA-I lectin, which causes increased permeability to its lethal cytotoxins across the intestinal epithelium.[29] The expression of PA-I is dependent on a QS signaling mechanism, a core system mediated by autoinducer molecules that control house-keeping and virulence genes in bacteria (Fig. 2). Although host cells are known to express receptors that bind bacteria for the purpose of activating the immune system, it is also feasible to think that bacteria themselves might possess specialized receptors that in turn recognize and respond to host immune activation by enhancing their virulence phenotype. Recent work by Wu et al has demonstrated that human interferon-γ binds to an outer membrane protein in *P. aeruginosa,* resulting in the expression of the QS-dependent PA-I lectin.[30] This study provides molecular evidence that certain opportunistic pathogens, such as *P. aeruginosa,* may have evolved a contingency-based mechanism to mount an effective countermeasure to immune activation by the host.[30]

A final example of novel host-pathogen communication occurs during pathogenic *Escherichia coli* infections. It has been proposed that QS autoinducers aid the disease process by allowing pathogenic bacteria to appropriately time expression of virulence factors that might activate a defensive immune response before the infection has progressed.[1] Furthermore, QS might also modulate host responses through regulation of commensal genes involved in gut colonization and host signaling.[10] The first evidence that QS could be involved in the regulation of virulence factors of gastrointestinal pathogens was found in enteropathogenic *E. coli* and enterohemorrhagic *E. coli* (EHEC) strains. Sperandio et al found that QS is responsible for regulation of intestinal colonization factors that play an important role in the pathogenesis of disease caused by these organisms.[31] Further studies indicate that QS is a global regulatory mechanism for basic physiological functions of EHEC as well as for virulence factors.[32] More recently, it was found that EHEC senses a bacterial autoinducer known as AI-3 (produced by EHEC and the normal intestinal microflora) and the host hormones epinephrine/norepinephrine to activate expression of several virulence factors (Fig. 2).[33] The discovery that these mammalian gut hormones mimic autoinducer signaling molecules implies a potential cross-communication between the bacterial QS system and the epinephrine host signaling system and highlights the complexity of the bacteria and host cells crosstalk.

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CHAPTER 9

Host-Microbe Symbiosis:
The Squid-Vibrio Association—
A Naturally Occurring, Experimental Model of Animal/Bacterial Partnerships

Margaret McFall-Ngai*

Abstract

Many, if not most, animals have specific symbiotic relationships with bacterial partners. Recent studies suggest that vertebrates create alliances with highly complex consortia of hundreds to thousands of prokaryotic phylotypes. In contrast, invertebrates often have binary associations, i.e., relationships with a population of a single bacterial species. In this chapter, the association between the Hawaiian sepiolid squid *Euprymna scolopes* and the marine luminous bacterium *Vibrio fischeri* is highlighted. This symbiosis offers a relatively simple, yet naturally occurring, association that can be experimentally manipulated. Studies of this system are providing insight into the precise mechanisms by which a beneficial animal-bacterial symbiosis can be established and maintained.

Introduction—The Context

Researchers in biomedicine are becoming increasingly aware that an understanding of evolutionary and ecological principals can provide great insight into the underlying dynamics of human health and disease. Humans, as all animals, are products of their evolutionary history, a basic feature that will be reflected in all aspects of their biology. This newfound awareness is likely to influence few groups of biomedical researchers as profoundly as those who study the relationships of microbes to their host animals. The evolution of animals occurred relatively late in earth's history as a patina over the continued evolution of the microbial world. Specifically, all animal body plans evolved at Cambrian explosion 540 million years ago in the context of marine environments with millions of bacterial cells in each milliliter of seawater. As such, from the beginning through the present day, animals have been interacting with microbes in a variety of ways. Thus, it is not surprising to find that the responses of present-day animals to microbes can be ancient responses, highly conserved over evolutionary history.

An example of this conservation can be found in the form and function of the immune system. Recent studies of innate immunity have demonstrated that all three major subkingdoms of the kingdom Animalia, i.e., the Deuterostomia, (e.g., vertebrates, sea squirts, urchins), Ecdysozoa (e.g., fruit fly and nematode worm) and the Lophotrochozoa (e.g., snails, squids, marine worms), share orthologous pattern-recognition receptors and elements of response pathways specific to interacting with the microbial world.[1] The invertebrates are highly diverse and evolutionarily successful, yet

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are thought to rely chiefly on the activity of the innate immune system to interface with microbes.[2] On the other hand, the gnathostome vertebrates, the divergence of which dates back to the very early diversification of the animals in the Paleozoic, have not only the innate immune system but also an adaptive immune system, the major components of which are conserved throughout all gnathostome classes of vertebrate, from bony fishes to mammals. Invertebrates are highly successful, have as a group every known life history strategy and show no higher morbidity or mortality to pathogenic infection than vertebrates. These traits suggest that the vertebrate adaptive immune system is not a 'better' interface with the microbial world, but rather an alternative strategy.

The differences in the immune systems of invertebrates and vertebrates have some reflection in trends of occurrence of their interactions with microbes. All animal cells, of course, have mitochondria, but the invertebrates very often have other binary, intracellular or extracellular symbioses. For example, approximately 11% of all insects have bacteriocyte symbioses wherein a monoculture of intracellular bacteria in the fat bodies (the liver equivalent) provides essential nutrients to the host;[3] also the widely studied associations of hydrothermal vent animals with sulfur oxidizing bacteria and coral hosts with their unicellular algae offer other examples of ecologically important binary, intracellular symbioses.[4] In contrast, vertebrates rarely, if ever, have beneficial binary, intracellular symbioses and extracellular binary alliances occur rarely and only among the fishes (e.g., the luminous bacteria-light organ symbioses in several families of marine fishes). Recent evidence suggests that vertebrates instead harbor highly complex consortia, a condition that appears to occur rarely in the invertebrates (exceptions—termites, cockroaches and their relatives)[5] and that the consortia of the vertebrate gut may profoundly affect the activity of the adaptive immune system.[6]

Despite these differences between the invertebrates and vertebrates, they share enough conserved elements in their interaction with microbes to render broad comparative studies of animal-microbe interactions compelling. If biologists are to obtain a reasonably accurate picture of the very basic mechanisms underlying the dynamics of animal-microbe relations, they must have a similar strategy to that employed by the developmental biologists, wherein a variety of models have been exploited (e.g., mouse, chick, frog, zebrafish, fruit fly, nematode, urchin, etc.). Each model has key features that have provided and continue to provide, the pieces of a complex set of puzzles. In the frontier of the field of animal-microbe interactions, a wide variety of model systems have been and are being developed. Among the vertebrates, most notable are the germ-free and gnotobiotic systems. The zebrafish and mouse are particularly powerful models in this group, as the genetics of host responses can be studied.[7-9] However, these systems are naturally consortial with hundreds to thousands of microbial partners; thus, genetic approaches on the microbes are likely to be limited in their ability to inform about the dynamics of the intact set of communities.

As simpler, binary associations, invertebrate symbioses ought to offer limitless opportunities, as they are diverse and abundant. However, many of these alliances are so tight that one or the other partner cannot be cultured outside of the symbiosis. This problem renders many of the invertebrate systems intractable as experimental models. However, recently several invertebrate symbioses, such as the nematode,[10-12] leech[13] and earthworm[14] associations with specific bacterial partners, are emerging experimental systems that hold great promise. Comparisons among these associations and comparisons of these systems with the vertebrate consortial symbioses should provide great insight not only into what is basic or conserved in animal-microbe associations but also into what processes create the diversity of symbioses.

The remainder of this chapter focuses on the squid-vibrio symbiosis. The intent is to provide an example of what is known to date about the degree complexity that can underlie the establishment, development and maintenance of a binary association. Studies of this system have been aimed at understanding how the symbionts are harvested from the environment, how specificity is achieved, how partner development is affected by their reciprocal interaction and how stability is achieved once a mature association is established. In addition, a key question asked in this system has been: how does the language of beneficial interaction differ from that of pathogenesis?

The Monospecific Squid-Vibrio Symbiosis as an Experimental System

The symbiosis between the Hawaiian sepiolid squid *Euprymna scolopes* and the marine luminous bacterium *Vibrio fischeri* has been studied for over 15 years as an unusually tractable experimental model for the study of animal-bacterial interactions at the interface of epithelial tissues and their associated, colonized lumina.[15] The partnership is highly specific in that only one bacterial species, against a background of thousands of other species in the ambient seawater, is capable of forming a stable relationship; i.e., in the absence of *V. fischeri*, no other environmental bacteria colonize host light-organ tissues. The binary nature of this symbiosis provides the opportunity to define the precise dialogue that occurs between the partners over the trajectory of their long-term relationship. As such, this symbiosis offers a complement to the studies of the dynamics of symbiotic associations that occur between mammalian hosts and their diverse and complex consortial microbiota.

As mentioned above, while many animals have binary associations with bacteria, rarely are both the partners easily culturable outside of the symbiosis.[4] Most often, the association is nutritionally based and either or both partners cannot withstand the aposymbiotic state (i.e., occurring in the absence of the native symbiont). In the squid-vibrio system, the light produced by *V. fischeri* is the principal product benefiting the host; no evidence exists that the bacterial symbionts provide nutrients to the host animal. The morphology of the light organ suggests that the host uses the light of the bacteria in antipredatory behavior called counterillumination, in which the host emits light as a camouflage. Thus, under laboratory conditions where no predators are present, the absence of the symbiont does not negatively affect the fitness of the host.

Several other characteristics of the host animal render it a suitable subject for studies of symbiosis. Male and female adult squids are easily obtained from the field and maintained in either running or recirculating seawater systems. A colony of 12-15 adult squids produces in excess of 50,000 juvenile squid/year that can be used for the experimental analyses of this symbiosis. Recently, an EST database of nearly 14,000 unique clusters has been created from juvenile light organ tissue and the cDNAs have been arrayed. These resources have expanded the studies of the host animal to the arena of genomic analysis.

The microbial light organ symbiont *V. fischeri*, a member of the gamma proteobacteria subgroup of Gram-negative bacteria, is among the best-understood marine bacteria due to its roles as a model both for bacterial light production and for symbiotic relationships with animals.[16,17] These two general facets of the biology of *V. fischeri* have spurred active research programs for almost three decades and the resulting studies have, perhaps surprisingly, converged in many respects with the field of pathogenic microbiology. For example, the phenomenon of quorum sensing, also known as autoinduction, whereby bacteria induce expression of particular genes only after achieving a critical cell-density, was first discovered through studies of *V. fischeri* luciferase regulation.[18] Subsequent to its discovery in *V. fischeri*, quorum sensing systems were found in several pathogenic bacteria, including *Pseudomonas aeruginosa*, *S. typhimurium*, *Vibrio cholerae* and *Helicobacter pylori*, where, at least in some cases, they contribute to the regulation of virulence factors.[19,20] Similarly, the interactions between *V. fischeri* and its animal hosts display several morphological and mechanistic parallels to host-pathogen associations.[21,22]

V. fischeri has also been well-studied, in part, because it is amenable to laboratory manipulations. It grows rapidly in liquid or solid culture (optimally doubling in <30 min), is prototrophic, tolerates a wide range of oxygen levels and is amenable to conjugally-or electrochemically-mediated transformation. Because genetic manipulations, particularly mutant analyses, constitute a powerful tool for dissecting the bacterial attributes that contribute to the squid-vibrio symbiosis, bacteriologists have developed a number of molecular and genetic tools for use with *V. fischeri*.[23-26] In additional, the recent sequencing and annotation of the *V. fischeri* genome has provided researchers not only with the information of the full complement of genes in the symbiont, but with a valuable source by which to compare genomes of *V. fischeri* with that of the pathogenic *Vibrio spp.*, such as *V. cholerae*.

In addition to the above-described favorable characteristics of each partner, several aspects of the symbiosis itself make it ideal for experimental analysis. These include the following characteristics—

(i) The time course of development of the symbiosis is relatively brief.[27] The animal host is colonized by the symbiont within hours of hatching and the symbiosis matures within a few days following the initial inoculation of host tissues. (ii) The infected and uninfected (aposymbiotic) animals can be compared directly. (iii) The extent of bacterial colonization can be quantified non-invasively and repeatedly, on the same animal by measuring light emission.[28] (iv) The light organ is accessible to dissection and observation during development. The size and anatomical relationships of the light organ tissues render real-time analysis of the progression of symbiosis by confocal microscopy an ideal approach (e.g., see Fig. 1). (v) The pores on the surface of the light organ extend into the light organ crypts, allowing experimentally introduced solutes (e.g., purified LPS, PGN, proteins, antibiotics or fluorochromes) to diffuse to the site of infection.[29-33] (vi) The juveniles are large enough to allow molecular analysis of the host's symbiotic tissues. And, (vii), the symbiosis can be studied intact, so that all naturally interacting systems (e.g., the epithelia and the innate immune system) are functional; therefore, the association provides a powerful complement to studies of host-bacterial interactions in cell culture.

Colonization of Host Tissues by *Vibrio fischeri* and Subsequent Symbiont-Induced Host Development

In the mature symbiosis, *V. fischeri* resides extracellularly within deeply invaginated epithelial crypts of the host squid's light organ.[34] Surrounding the bacteria-rich epithelial core are tissues that serve to direct and diffuse the bacterial luminescence, as well as control the intensity of the emitted light. As in most coevolved animal-bacterial symbioses, including those of humans, the squid-vibrio association is horizontally transmitted between generations, i.e., the host acquires the symbiont population anew each generation. During embryogenesis, the host animal develops a set of tissues that prepares it for immediate interaction with environmental *V. fischeri* cells when it hatches from the egg (Fig. 1).[35] Specifically, a bilaterally symmetrical nascent light organ is developed that bears, on each side, a complex, superficial, ciliated epithelium. This tissue is involved in potentiating the colonization of host tissues by the symbionts.[36] In the middle of each ciliated field, at the base of two extended epithelial appendages, are three pores, the sites of eventual entry of the *V. fischeri* cells. During colonization, the bacteria enter these pores, travel up ducts and invade three independent crypt spaces. The population of *V. fischeri* cells that has entered the crypts then grows to fill the crypt spaces within twelve hours.[37] Restricted to these anatomical sites throughout the life history of the host, the bacterial symbionts interact with two cell types: the crypt epithelia and migrating phagocytes, or hemocytes, which sample the crypt spaces.[37,38] The host controls the symbiont population by a daily venting of 90-95% of the bacterial culture from the light organ pores out into the surrounding seawater; growth of the remaining 5-10% of the population over the subsequent 12 h fully recolonizes the organ each day.[38,39]

Studies of the colonization process in the squid-vibrio symbiosis have revealed that hatching into environmental seawater induces the cells of this ciliated epithelium to shed mucus that is focused, by the activity of the cilia, into masses above the light organ pores[36] (Fig. 1B and C). The symbionts aggregate in this mucus and, after some residence time as an aggregate (2-3h), migrate to pores on the surface of the organ, through the ducts and into their final place of residence in the crypt spaces (Fig. 1D-G). As *V. fischeri* aggregates in the mucus, a 'winnowing' occurs during which ever-increasing specificity results in competitive dominance of *V. fischeri*.[15] Specifically, whereas both Gram-positive and negative bacteria are capable of inducing host mucus secretion, only Gram-negative bacteria adhere to this mucus and only living Gram-negative bacteria form tight aggregations. In the absence of *V. fischeri* other Gram-negative bacteria will aggregate in the mucus, but when *V. fischeri* cells are present at their normal ratio to other environmental bacteria (i.e., 1:10,000), after a 3-h incubation period, the aggregate contains *V. fischeri* cells exclusively.[40] These data suggest that *V. fischeri* is, by some means, a competitive dominant in the host-secreted mucus. Analyses of this phenomenon have indicated that this facility of the symbiont is most likely due to an enhanced ability to occupy sites in the mucus (e.g., to better adhere to the matrix or resist antimicrobial substances in the mucus), rather than competitive dominance for a resource; growth

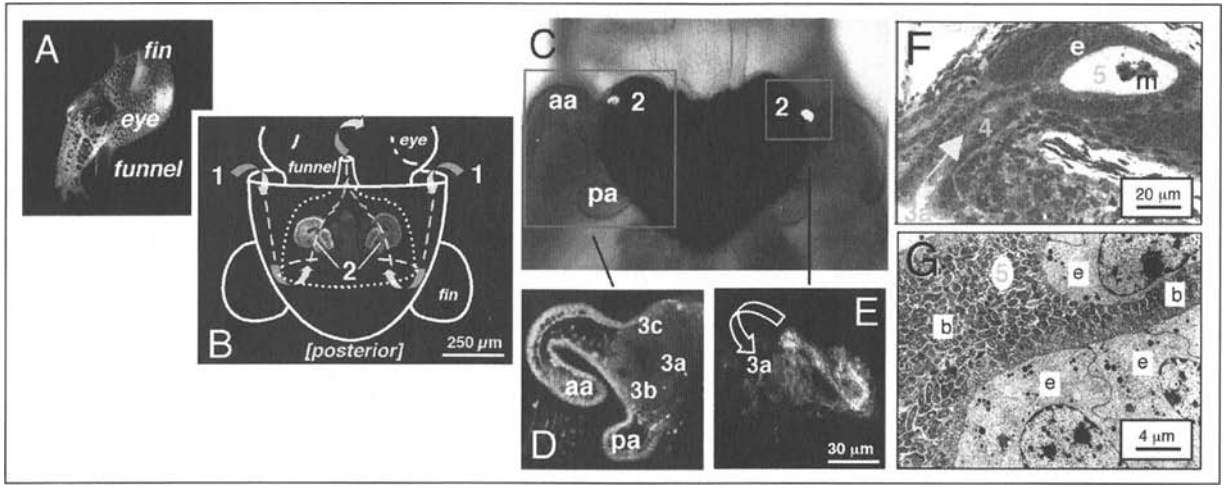

Figure 1 The pathway of colonization of the *E. scolopes* light organ by *V. fischeri*. A) The host squid E. scolopes, which as a newly hatched juvenile averages 3 mm in total length, is colonized by *V. fischeri* cells within hours following emergence from the egg. B-G) The path of the symbiotic bacteria (designated 1-5) that brings them from the surrounding environmental seawater into their eventual colonization site in the host epithelium-lined crypts. B) A diagram of the host's body cavity viewed ventrally. A confocal micrograph of the light organ is projected in this cutaway. Seawater that contains potential symbionts (1) is brought to the site of susceptible host tissues by the normal ventilatory movements of the host (arrows/dashed lines). Symbionts gather (2) in a biofilm of host-derived mucus, the production of which is stimulated by exposure of the host to peptidoglycan from environmental bacteria. C) A differential-interference-contrast image of the light organ, following a 2-3 h exposure of the host to water containing GFP-labeled *V. fischeri* (grey aggregates to the left and right of '2'). The activity of two lateral, superficial fields of ciliated epithelial cells, with their anterior (aa) and posterior appendages (pa) serves to suspend the symbiont-mucus aggregate above the organ surface (2). D) A confocal image of one-side of the light organ showing the three pores (3 a-c) through which the bacteria will enter host tissues. E) After a residence time in the aggregates of a few hours, the symbionts migrate (arrow) toward the pores (at arrowhead) and into the ducts leading to the internal crypt spaces. In this confocal image-GFP-labeled *V. fischeri* (bright regions) are seen in association with the host-shed mucus, which has been labeled with a fluorescent lectin (dimmer grey). F) A light micrograph of a cross section through the uncolonized juvenile organ. Symbionts travel into each pore (3a depicted here) and through each long duct (4, arrow), into crypt spaces (5), where the bacterial cells encounter the crypt epithelial (3) and host-macrophage-like cells (m) with which they will interact throughout the life of the host. G) A transmission electron micrograph of *V. fischeri* (b) colonizing the crypt spaces. [For review see Nyholm & McFall-Ngai, 2004].

of the bacterial population is minimal in the aggregates.[40] Following the migration of an aggregate into the crypts (Fig. 1E and F), subsequent aggregates are formed in a similar fashion and continue to form and migrate into the crypts over the first 24 h post hatching.[41] However, between 24 and 36 h following colonization, *V. fischeri* cells in the crypts cause the secretion of mucus from the superficial epithelium to cease and no additional aggregates are formed.

Within hours of the initial crypt colonization, the symbiont cells induce a series of developmental changes both in the crypt cells with which they directly interact and in the remote superficial epithelial fields of the organ, which have facilitated colonization (Fig. 2); for review see.[15] The most dramatic of the developmental changes is the complete regression of the superficial field of epithelial cells.[27] This process is characterized by a hemocyte trafficking into the blood sinuses of the ciliated epithelia and apoptosis of the cells of this field. The ducts change both anatomically and biochemically in response to interaction with the symbionts. Nitric oxide production, which is high in the ducts of aposymbiotic animals, is attenuated with the onset of the symbiosis and changes in the actin cytoskeleton of the ducts results in their constriction. The epithelial cells that line the crypts, i.e., those cells that will interact with the symbionts persistently, exhibit an increase in the density of their microvilli, as well as swell 4-fold in cytoplasmic volume, in response to the direct interactions with *V. fischeri*.

Microbe-Associated Molecular Patterns of *V. fischeri* and Host Responses to These Molecules During the Early Stages of the Symbiosis

The conserved molecules of the bacterial envelope, particularly components of the lipopolysaccharide (LPS) of the outer membrane and the peptidoglycan (PGN) of the cell wall, signal play a critical role in the early stages of the squid-vibrio symbiosis. The activities of this class of bacteria-specific molecules, examples of microbe-associated molecular patterns (MAMPs; 53, 62), are most often associated with and best understood in the onset and progression of bacteria-induced disease.[42,43] In the pathogenesis, LPS and PGN can work either alone or in concert[44-47] and are known to play central roles in host response (e.g., in the mediation of septic shock).[48,49]

The influence of MAMPs begins immediately upon hatching of the juvenile host. Mucus secretion by cells of the nascent light organ is induced by the exposure of the animal to the PGN that has been shed by the Gram-positive and Gram-negative environmental bacteria.[41] In addition, the morphogenetic process that results in the loss of the superficial ciliated epithelium is due to the synergistic activity of *V. fischeri*-shed PGN and LPS derivatives.[31] Beginning at about 2 following initial exposure to *V. fischeri*, i.e., coincident with aggregation of symbiont cells in host-secreted mucus, the migration of hemocytes into the blood sinues of the superficial epithelial field of cells occurs in response to a *V. fischeri*-shed PGN fragment, specifically the tetrapeptide fragment of PGN that has been most widely called 'tracheal cytotoxin' or 'TCT'. TCT was first described in *Bordetella pertussis* infection where it causes the epithelial cell disruption characteristic of that infection (Koropatnick and McFall-Ngai, Luker et al., 1993[50a]). At about 6 h following initial exposure of the host squid to environmental *V. fischeri*, when the symbionts are traveling through the ducts, the first apoptosis events triggered in the cells of the superficial epithelium in response to exposure to *V. fischeri* lipid A, a component of LPS.[32] Early characterizations of this phenomenon showed that the numbers of hemocytes trafficking into this field, as well as the numbers of epithelial cells undergoing apoptosis, peak at ~12 h, but the processes continue throughout the regression process. Around this 12 h time point, the lipid A and TCT shed by *V. fischeri* in the crypts send an irreversible signal that results in the full regression of the superficial field, a process that requires 4 d to complete. Specifically, when animals are cured of symbionts before 12 h, or exposed to TCT and LPS for less than 12 h, the field does not regress; however, if they are cured at or after 12 h of exposure, the full 4-d program continues unabated.[31,33]

The recognition and response system to MAMPs is also highly conserved among animals responding to pathogens and recent studies of the squid-vibrio system suggest that these same

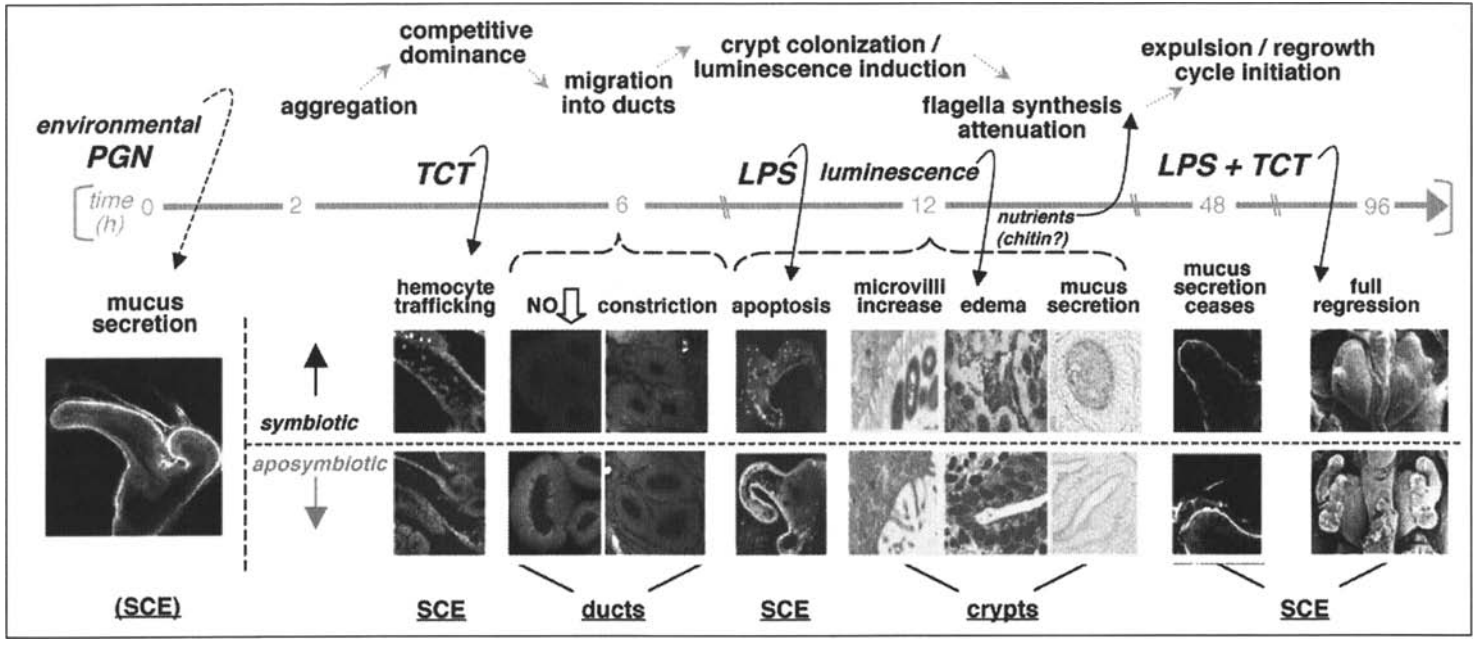

Figure 2. Microbe-associated molecular patterns (MAMPs) as signaling molecules in early infection by *V. fischeri*. The host and symbiont undergo a complex reciprocal dialogue during the first days of colonization that leads to dramatic changes in both partners. In this depiction, events and structures listed above the line illustrate responses of *V. fischeri*, those below indicate the responses of *E. scolopes*. For *E. scolopes*, cell and tissue characteristics in the presence (symbiotic, above the dashed line) or absence (aposymbiotic, below the dashed line) of *V. fischeri* are indicated. Curved arrows indicate direction of signals (italics) communicated between the partners. Host tissues in which the events occur are underlined. LPS, lipopolysaccharide; h, hours following hatching into natural seawater; NO, nitric oxide; PGN, peptidoglycan; SCE, superficial ciliated epithelium; TCT, tracheal cytotoxin, a PGN derivative.

elements are involved in mediating beneficial symbiosis as well.[50-53] *E. scolopes* shares with other animals, including vertebrates and invertebrates,[52,54-57] a series of binding proteins and receptors ('pattern recognition receptors' or 'PRRs'), response pathways and effector molecules. Analysis of the squid light-organ EST database revealed that the light organ expresses during early development three orthologs of the LPS-binding proteins (LBPs), four orthologs of the peptidoglycan recognition proteins (PGRPs) and one ortholog of a Toll-like receptor (TLR).[58] Studies of these molecules in other systems have demonstrated that they can function as receptors themselves, or adaptors that interface the bacterial ligand with its cognate receptor molecule and they can act as monomers, or homo- or heteromultimeric complexes.[59-61] In addition, some of these PRRs, most notably certain PGRPs and LBPs, act as bacteriostatic or bactericidal agents.[57,62,63] The roles of these molecules in mediating specificity of the squid-vibrio symbiosis and in responding to the bacterial MAMPs during development remain to be determined.

Biologists have also identified several conserved response pathways to these receptor-ligand interactions, most notably the NF-kB, JNK and p38 MAP kinase pathway and JAK-STAT pathways.[64,65] In each case, the response induced by the ligand-receptor interaction leads to changes in gene transcription associated with prokaryotic-eukaryotic cell-cell interaction, such as genes that mediate production of antimicrobial agents (e.g., nitric oxide and antimicrobial peptides) or those involved in cytokine production. Thus far, orthologs of proteins in the NF-kB, p38 MAP kinase and JAK-STAT pathways have been found in the EST database of the *E. scolopes*. Expression of these genes during early development suggests that they may be involved in response to interactions with *V. fischeri*, perhaps with *V. fischeri* MAMPs. As with the PRRs, an understanding of the role of these pathways in this symbiosis remains to be elucidated. One interesting avenue will involve how these molecules and pathways are used to manage a beneficial symbiosis and how this differs from the way this animal uses these very same elements to control bacterial pathogenesis.

Luminescence—The Central Feature of the Symbiosis

The application of microbial genetics has revealed a number of *V. fischeri* characters that are required for normal symbiosis. These important aspects of the association have recently been reviewed, so will not be mentioned here.[66] However, one principal feature of the symbiosis, i.e., luminescence, will be covered briefly.

In every symbiosis, the host and symbiont(s) have a 'currency' of exchange that defines the partnership. In the squid-vibrio association, the host provides nutrients for the bacteria and, in exchange, the bacteria produce light that the host uses in its behavior. One might suspect that the bacteria would 'cheat' and not do their part, as luminescence production imposes a metabolic cost to the bacterial cell. However, studies of the association have suggested that the host has mechanisms to ensure that the bacteria are luminous.[67] Mutants in the *luxA* gene, which encodes one of the subunits of the symbiont's luciferase, are incapable of producing luminescence. Such mutants can colonize the light organ initially, but fail to persist, i.e., following the first day, their numbers in the host light organ decline. These mutants also fail to induce the normal swelling of the light-organ crypt epithelial cells that is induced by wild-type *V. fischeri*. Experimental manipulation of the system has indicated that these mutants are defective in obtaining nutrients from the host (E. Ruby, pers. comm.). Although it has not been shown unequivocally, these findings would suggest that the host cell-swelling phenotype is involved in the provision of nutrients to the symbiont population.

How the host cells perceive symbiont luminescence is not understood. However, the nature of the luminescence reaction presents two possibilities. In this reaction, oxygen is consumed and light is produced and all other substrates are recycled.[68] This chemistry suggests that the host perceives either the light itself and/or a change in the oxygen tension in the crypts. An analysis of the light organ EST database revealed a surprising finding—the organ expresses proteins that may perceive light, including the blue-light receptor protein, cryptochrome, as well as many of the components of the visual transduction cascade, including rhodopsin, rhodopsin kinase and arrestin, which are generally eye specific. The expression of these proteins suggests that the light organ tissue has the

biochemical potential to perceive bacteria-produced light. In addition, a large number of proteins associated with the amelioration of oxidative stress are also expressed. Obviously, numerous questions are raised by these observations and resolving the mechanism of light production will require extensive further research on the system. However, these data suggest that an unraveling of how luminescence is controlled in this symbiosis is within reach.

Summary

Studies of the squid-vibrio association have revealed that the partners undergo a very complex reciprocal dialogue that promotes the successful colonization of host tissues. Most notably, experiments with the system have demonstrated that many of the interactions of this beneficial association involve features that have been previously ascribed principally to pathogenesis. Most notably, the bacterial partner presents to the host cells lipopolysaccaride and peptidoglycan derivatives, specific fragments that have been labeled as 'toxins' that damage animal cells and induce inflammation in other systems. However, in the dynamics of the squid-vibrio system, these molecules behave as morphogens. The bacteria use these molecules to communicate to the host partner that symbiosis is established and development can ensue. The developmental program transforms the organ from a colonization morphology to one that associated with the mature, functional symbiosis. Also in common with pathogenesis is the induction in this symbiosis of apoptosis and cellular edema, as well as the involvement of toxic oxygen and nitrogen species. Taken together, these findings demonstrate that many of the molecular responses of animals to their bacterial symbionts are not only ancient, but also that they can be shared by beneficial and pathogenic associations.

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CHAPTER 10

The "Microflora Hypothesis" of Allergic Disease

Andrew Shreiner, Gary B. Huffnagle and Mairi C. Noverr*

Abstract

Predisposition to allergic disease is a complex function of an individual's genetic background and, as is the case with multi-gene traits, environmental factors have important phenotypic consequences. Over a span of decades, a dramatic increase in the prevalence of allergic disease in westernized populations suggests the occurrence of critical changes in environmental pressures. Recently, it has been shown that the microbiota (i.e. microflora) of allergic individuals differs from that of non-allergic ones and that differences are detectable prior to the onset of atopy, consistent with a possible causative role. Features of the westernized lifestyle that are known to alter the microbiota, such as antibiotics and diet, are also associated with allergy in humans. In this chapter, we discuss the "Microflora Hypothesis" for allergy which predicts that an "unhealthy" microbiota composition, now commonly found within westernized communities, contributes to the development of allergy and conversely, that restoring a "healthy" microbiota, perhaps through probiotic supplementation, may prevent the development of allergy or even treat existing disease. In testing this hypothesis, our laboratory has recently reported that mice can develop allergic airway responses if their microbiota is altered at the time of first allergen exposure.

Introduction

Allergic diseases are manifested by inappropriate immune responses to harmless foreign materials in those with a genetic predisposition. Recent decades have witnessed a substantial rise in the prevalence of allergies in Westernized communities, in contrast to the stable, low prevalence common in less developed areas.[1] This period is so brief as to suggest that fresh environmental pressures, as opposed to genetic alterations, underlie this phenomenon.[2-4] Increasingly, epidemiological data demonstrates that the composition of the gastrointestinal microbiota is associated with the allergic phenotype. Other studies suggest that multiple features of the Westernized lifestyle, including characteristic dietary and antibiotic use patterns, affect the composition of the microbiota and may in that manner contribute to the incidence of allergic disease. Our laboratory has recently reported that mice can develop allergic airway responses to allergens if their endogenous microbiota is altered at the time of first allergen exposure. These experimental and clinical observations are consistent with other studies demonstrating that the endogenous microbiota plays a significant role in shaping the development of the immune system and accumulating data that supports a role for the microbiota in maintaining mucosal immunologic tolerance long after post-natal development. To that end, there is considerable interest in therapies that employ the administration of probiotic bacteria that provide beneficial effects to the host, including anti-inflammatory properties. The

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"Microflora Hypothesis" states that critical interactions between the gastrointestinal microbiota and the immune system can impinge upon immunological mechanisms resulting in tolerance or allergy.[5] We predict that an "unhealthy" microbiota composition, now commonly found within Westernized communities, contributes to the development of allergy and conversely, that restoring a "healthy" microbiota, perhaps through probiotic supplementation, may prevent the development of allergy or even treat existing disease.

The United States population experienced an increase of 75% in the prevalence of asthma from 1980 to 1994.[1] Geographically distant countries with similar lifestyles, such as the United Kingdom[6] and Australia, reported comparable increases in asthma prevalence during this period of time. Currently, in the United States, Canada, United Kingdom, Ireland, New Zealand and Australia, the prevalence of allergic airway disease among 13-14 year old children is among the highest in the world and ranges from 22-32%.[7,8] In response to these developments, the International Study of Asthma and Allergies in Childhood (ISAAC) was initiated with a stated goal to determine the prevalence of allergic disorders in children living throughout the world using standardized criteria, increasing comparative value. The Phase I survey of 450,000+ children in 56 countries revealed stark geographic differences in prevalences.[9] The pattern and magnitude of variation throughout the world suggest that environmental factors are critical to the development of allergies in childhood. Upon further evaluation of the ISAAC and the European Community Respiratory Health Survey (ECRHS) of asthma prevalence in adults, several trends became apparent including (1) an increase in allergy prevalence throughout the world, (2) a pattern of asthma prevalence where it is more common in Westernized countries and less common in developing countries and (3) an increase in prevalence as countries Westernize or as communities urbanize.[2]

After comparing atopic disease prevalence between white and Aboriginal families in Saskatchewan, Gerrard and coworkers suggested in 1976 that atopic disease may be the price paid for freedom from infectious diseases.[10] The "hygiene hypothesis" for allergy gained widespread attention after Strachan proposed in 1981 that a decrease in the episodes of early life infections due to increased cleanliness in the home and decreased sibship may underlie the increasing incidence of hay fever noted in the UK.[11] This notion that an early deprivation of infection may lead to immune dysregulation was also invoked as an explanation for the concomitant rise in the prevalence of autoimmune disorders seen in Westernized countries during recent decades.[12] The depth of the collective understanding of immune regulation has been greatly increased during the intervening period through revelatory work on the biology of regulatory T-cells (Treg) and certain dendritic cell (DC) subsets with regulatory function. Furthermore, the identification of pattern recognition receptors (PRRs) including the Toll-like receptor (TLR) family has further illuminated the expansive ability of cells of the immune system to interact with the microbial world in order to achieve an appropriate immune response. This type of information regarding immune regulation taken together with accumulated epidemiological evidence concerning the association between environmental factors and diseases of immune dysregulation, has lead to a refashioning of the original "hygiene hypothesis". A revised understanding of the hypothesis should encompass the idea that alterations in certain interactions with the microbial world in general and not infections in particular, in Westernized countries has lead to improper maintenance of immune regulation resulting in an increase in the incidence of allergic and possibly autoimmune disease.[13,14]

The focus of this chapter is on an alternative interpretation of the data supporting the "Hygiene Hypothesis." The "Microflora Hypothesis" for allergy proposes that perturbations in the gastrointestinal microbiota due to aspects of the modern lifestyle pervasive in Westernized countries have disrupted the normal microbiota-mediated mechanisms which program immunological tolerance in the mucosa, leading to an increase in the incidence of allergic disease. Other groups have formulated arguments that agree in principle with the central tenet of the "Microflora Hypothesis." Rook and Burnet suggest that nonpathogenic microbes which have shared human's evolutionary past are recognized by the innate immune system and instruct the development of immunoregulatory responses that inhibit allergic disorders, autoimmune disease and inflammatory bowel disease.[15,16] Bjorksten contends that no major risk factors leading to the development of allergies have been

indentified, but rather that recent evidence indicates a deficit in the protective influence of interactions with the intestinal microbiota during infancy may be leading to the increase in allergic disease.[17] There is a significant amount of epidemiologic and clinical data supporting this altered microbiota hypothesis. These include correlations between allergic airway disease and (1) altered fecal microbiota, (2) antibiotic use early in life and (3) dietary changes over the past two decades (for a review see 18). Our laboratory has recently demonstrated that mice can develop allergic airway responses to allergens if their endogenous microbiota is altered at the time of first allergen exposure.[19] In contrast, mice with normal microbiota do not develop allergic responses upon airway exposure to the allergens. Researchers in Finland have acknowledged the role of the microbiota in influencing allergic disease and have focused on the potential therapeutic effect of probiotic microbes in restoring beneficial interactions between the immune system and the microbiota.[20] Successful probiotic therapy for allergy with single organisms or simple combinations would make an important "proof of principle" demonstration that the microbiota can exert an influence on allergic disease. Of course most importantly, such a demonstration would provide an exciting, alternative and readily accessible treatment for a collection of diseases that take an enormous toll around the world. Probiotic therapy for inflammatory disorders including atopic disease is the subject of a chapter by Kalliomaki et al in this book, so it will not be discussed in detail here.

The Epidemiological Association between Allergies and Microbiota Composition

A direct link between the microbiota composition and atopic disease underpins the "Microflora Hypothesis" of allergy. A number of recent studies directly compared the microbiota between allergic and non-allergic populations (Table 1). Several notable findings recurred in separate studies. First, significant differences exist in the microbiota composition between allergic and non-allergic individuals based on selective growth or molecular analysis of the bacterial flora.[21-32] Second, prospective studies detected differences in the microbiota prior to the development of atopy and/or allergy.[22,24] Third, while there exist numerous differences in the particular associations made between the microbiota and allergy in this collection of reports, several common findings or themes did arise. For instance, a negative association with allergies was found for lactic acid bacteria such as *Bifidobacteria*, while a positive association was made with Clostridia.[21-25,28-32]

In a series of publications, Bjorksten and coworkers describe the analyses of fecal microbiota with regard to allergic phenotype in patients selected from large study groups recruited in countries with a high (Sweden) or low (Estonia) prevalence of allergic disease. While the Swedish infant population had increased cumulative incidences of allergy (atopic dermatitis) and atopic sensitization (positive skin-prick test (SPT)) during the first two years of life, allergic children from either country had a similar microbiota compsition that differed significantly from the non-allergic population, suggesting that the alterations of the microbiota may be the underlying factor in allergic disease regardless of other environmental differences that may exist between these two countries.[21,33] Allergic children had reduced measures of colonization with *lactobacilli* and *bifidobacteria* and increased counts of certain aerobes, including coliforms or *Staphylococcus aureus*. In another study, lower bifidobacteria counts were also seen in infants with atopic dermatitis, however they actually had lower total counts of aerobes as well with a reduced frequency of gram-positives within the aerobic population.[25] In a detailed analysis of fifty bifidobacterium strains, allergic infants had a distinct pattern more typical of adults with higher levels of *Bifidobacterium adolescentis* compared to non-allergic infants that had higher levels of *B. bifidum*.[23] Similar findings were published in a separate report from the same authors.[26] Atopic (positive SPT) and recurrent wheezy (>3 episodes in the first year) infant cases had significantly higher levels of IgG specific for *Clostridium difficile* than their non-atopic, nonwheezy matched controls.[31] Molecular analysis of metabolic byproducts demonstrated that 13-month-old allergic infants had significantly higher levels of i-caproic acid that is strongly indicative of the presence of *Clostridium difficile*.[30] Proportions of bifidobacteria were lower and clostridia were higher in allergic 5-year-old children, indicating that differences in the microbiota are not restricted to the period of infancy.[28] These studies and others, indicate

Table 1. The epidemiological association between allergies and microbiota composition

		Subjects			Allergies		Experiments		Microflora Associations with Allergic Disease[f]		
Year	**Design[a]**	**Number[b]**	**Residence**	**Age**	**Disease[c]**	**Diagnosis[d]**	**Sample**	**Assay[e]**	**Negative[g]**	**Positive[h]**	**Reference**
1999	C-C	27-35	Estonia + Sweden	2 yr	AD (FA)	clinical and SPT	feces	microbiol	Lactobacilli, Bifidobacteria, CONS, Anaerobes, Bacteroides	Aerobes, Coliforms, *S. aureus*, Bacteroides	21
2000	C-C	25-47	Sweden	12 mo	AD, asthma, FA	clinical and SPT	feces	molecular	propionic, butyric, i-butyric, i-valeric and valeric FA	i-caproic FA[i]	30
2001	Pro	44 (18)	Estonia + Sweden	0-2 yr	AD (FA)	clinical and /or SPT	feces	microbiol	Enterococci, Bifidobacteria, *Bacteroides*	*S. aureus*, Lactobacilli, Clostridia	22
2001	C-C	4-6	NR	2-7 mo	AD and FA	clinical	feces	microbiol	*Bifidobacterium bifidum*	*Bifidobacterium adolescentis*	23
2001	Pro (FH)	76 (22)	Finland	0-12 mo	atopy (+/– AD/FA)	SPT (clinical)	feces	microbiol, molecular genetic	Similarity (%) of bacterial FA profile	Clostridia	24
2001	C-C	27-10	Finland	0-14 mo	AD (FA)	clinical and SPT	feces	microbiol, genetic	Gram-positive species within aerobes, *S.viridans*, Clostridia[i]	Lactobacilli/ enterococci	25
2001	C-C	7-6	NR	2-7 mo	AD	clinical and SPT	feces	microbiol	*Bifidobacterium bifidum*	*Bifidobacterium adolescentis*	26
2002	C-C	10-10	UK	12 mo	atopic wheeze	clinical and SPT	blood	molecular		*C. difficile*-specific IgG	31
2003	C-C	30-68	Japan	<20 yr	AD	clinical	feces	microbiol	*Bifidobacterium*	*Staphylococcus*	32

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Table 1. Continued

		Subjects			Allergies		Experiments		Microflora Associations with Allergic Disease[f]		
Year	Design[a]	Number[b]	Residence	Age	Disease[c]	Diagnosis[d]	Sample	Assay[e]	Negative[g]	Positive[h]	Reference
2005	C-C	33-33 (8-8)[k]	UK	3-5 yr	atopic wheeze (AD)[k]	clinical and SPT	feces	genetic	(Bifidobacteria)[k]		32
2005	C-C	19-19	Estonia	5 yr	AD, asthma, allergic rhinits	clinical and SPT and/or IgE	feces	microbiol	Bifidobacteria	Clostridia	28
2006	C-C	21-28	Singapore	~3 yr	AD	clinical	feces	microbiol, genetic	*Bifidobacterium* spp., *Clostridium* spp.	Lactic acid bacteria, enterococci	29

[a]C-C = Case-Control; Pro = Prospective; FH = inclusion based on Family History of allergy. [b]For C-C, 'number of cases-number of controls'; for Pro, 'total subjects (allergic subjects)'. [c]AD = atopic dermatitis or eczema; FA = food allergy; (FA) = food allergens included in skin-prick test. [d]SPT = positive skin-prick test; IgE measured by ELISA. [e]microbiol = various microbiological culture techniques. [f]Significant associations ($p < 0.05$) listed between allergic disease and microflora, generally in terms of microbial counts and/or the frequency of colonizaition with microbe (associations made with characteristic bacterial fatty acids (FA), or with IgG antibodies specific to bacteria as indicated). [g]Bacteria (or markers) are negatively associated with allergic subjects or positively associated with healthy control subjects. [h]Bacteria (or markers) are positively associated with allergic subjects or negatively associated with healthy control subjects. [i]i-caproic fatty acid is "associated with the presence of *C. difficile*". [j]Associations made between disease severity and bacterial counts. [k]Within the study of 33 pairs of atopic, wheezy cases and controls, only the subset of 8 atopic dermatitis cases exhibited significant differences with their controls.

that alterations in the microbiota composition exist in allergic populations. It is also clear that careful, sophisticated examinations of the flora are necessary to uncover the subtleties of this complex population in order to better define the "unhealthy" composition that may contribute to atopic disease.

The aforementioned studies compared the microbiota population in healthy and allergic populations. In order to explore the possibility of a causative role for altered microbiota in the development of atopic disease, several groups performed prospective studies on the flora of infants prior to the development atopy or atopic disease. In one study, the fecal microbiota composition of allergic and non-allergic infants was measured at various timepoints in the first two years of life in children with a clear family history of allergy.[22] As early as one week, differences in the microbiota were measured in those that would and would not develop allergies later in life. Indeed, the authors note the differences were less pronounced between the two groups after the first month of life. At one week, the prevalence of colonization with enterococci and *bifidobacteria* was lower in future-allergic infants and the difference in bifidobacteria colonization was maintained throughout the first year. At 3 months, allergic infants had significantly higher counts of Clostridia. In another study of infants at risk of developing atopic disease, alterations in the fecal flora were apparent before atopic sensitization was detectable.[24] Atopic infants had an early trend to lower *bifidobacteria* and a significant increase in clostridia. The connection between alterations in the flora and an allergic immune response was furthered by a correlation between total serum IgE and counts of clostridia. These studies indicate differences in the microbiota exist between allergic and nonallergic populations very early in infancy prior to the manifestation of allergy. Basic research studies will help determine if particular strains or combinations can promote allergic responses in susceptible animals.

Associations between Features of the Westernized Lifestyle and Allergic Diseases

Several features of the westernized lifestyle affect the composition of the gut microbiota and may in that manner contribute to the manifestation of allergic disease. The GI tract of infants is sterile at birth but colonization begins upon delivery. GI colonization involves a series of ecological successions influenced by dietary changes and host development (for a review see ref. 35).[34] Major factors affecting the nature of the early microbial populations include antibiotic use in the mother, mode of delivery and type of infant feeding. Reciprocal interactions during infancy between the microbiota and host immune system serve not only to generate a "healthy" microbiota but also to stimulate the proper development of the immune system. Ultimately, control of the microbiota composition depends on multiple factors, including microbe-microbe interactions (competitive exclusion), metabolic competition, host factors and innate and adadptive host defenses.[36-38] Antibiotics and diet can dramatically affect the stability of the microbiota populations.

The major effects of antibiotic treatment on the microbiota are the direct effect of killing a large proportion of the microbiota and the indirect effect of decreasing colonization resistance within the GI tract. Colonization resistance is a multi-faceted mechanism whereby obligate anaerobic microbiota inhibit the overgrowth of potentially harmful exogenous or endogenous microbes. The end result of a reduction in colonization resistance can either be clinically asymptomatic (leading only to an imbalance in the microbiota), localized symptomatic (e.g., diarrhea) or systemic symptomatic (disseminated infection).[39] Interestingly, changes in the microbiota populations can persist months after cessation antibiotic therapy and can result in long-term decreases in beneficial anaerobic organisms (*Bifidobacterium, Lactobacillus, Bacteroides*) and increases in potentially harmful microbes (gram negative aerobic enteric bacteria, the anaerobe *Clostridium dificile* and the yeast *Candida albicans*) (for a review see ref. 18).[40-48]

National trends of antibiotic use vs. incidence of allergic disease in industrialized (high atopy, high antibiotic use) vs. developing countries (low atopy, low antibiotic use) suggest a possible relationship.[2-4] A number of studies have identified a correlation between early antibiotic use in children and the subsequent development of allergy/asthma (Table 2). However, it is not

Table 2. The epidemiological association between early antibiotic use and allergies based on the odds ratio for developing allergic disease after antibiotic treatment

		Subjects			Evaluation Criteria[b]		Data Collection[c]			Odds Ratio[e]				Common Features		
Year	Design[a]	Number	Residence	Description	Antiobiotic Treatment	Allergy Diagnosis	Antibiotic	Allergy	Population Stratification[d]	Atopy	Dermatitis	Rhinitis	Asthma	Early Antibiotic[f]	Multiple Antibiotics[g]	Ref.
1998	Retro (PB-BC)	1934	UK	General	<2 yr	≤12 yr	MR	MR		*2.07	*2.04[h]	*2.04[h]	*3.19[h]	Y	ND	49
1999	Retro (PB)	5067+	Germany	General	<3 yr	≤5–11 yr	PR	PR (SPT)	1-2 antibiotic courses (AB)	0.96	*1.26	1.12	*1.64	ND#	Y	50
									≥6 AB	0.92	*1.57	*1.58	*7.95			
1999	Retro (CSS)	456	New Zealand	Anthroposophic (RS)[i]	<1 yr	≤5–10 yr	PR	PR		ND	1.23	1.99	*2.74	Y	Y	57
2000	Retro (PB)	742	Belgium	General	<1 yr	≤7–8 yr	PR	PR (SPT)	no FH hay fever	0.7	1.2	1.1	1.2	ND#	ND	58
		438							FH hay fever	1.6	*1.6	*2.8	*2.3			
2001	Pro (PB-BC)	939	Germany	General (38% atopy risk factors)	<3 yr	≤7 yr	PR	PR		ND	ND	ND	1.08[j]	ND#	N[j]	56
2001	Retro (PB)	2512	Germany	General	any	≤5–14 yr	PR	PR (SPT)	1 AB	0.9	1.3	2.3	1.1	Y	Y	51
									>5 AB	1.0	*2.3	*3.5	*7.4			
2002	Pro (FH)	498	US	FH of atopy	<1 yr	≤5 yr	PR	PR	1 AB	ND	0.9	0.9	0.5	ND#	N	61
									≥2 AB		1.1	0.7	0.8			
2002	Pro (PB-BC)	29,238	UK	General	<1 yr	≥1 yr ≤11 yr	MR	MR	1 AB	ND	*1.22	1.14	*1.26	ND#	N/Y	52
									>4 AB		1.01	1.14	*1.99			
2003	Retro (C-C)	7098-7098	UK	hayfever-control	<1 yr	≤5+ yr	MR	MR	1 AB	ND	ND	0.98	ND	N	N	62
									≥3 AB			0.82				

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Table 2. Continued

Year	Design[a]	Subjects: Number	Subjects: Residence	Subjects: Description	Evaluation Criteria[b]: Antiobiotic Treatment	Evaluation Criteria[b]: Allergy Diagnosis	Data Collection[c]: Antibiotic	Data Collection[c]: Allergy	Population Stratification[d]	Odds Ratio[e]: Atopy	Odds Ratio[e]: Dermatitis	Odds Ratio[e]: Rhinitis	Odds Ratio[e]: Asthma	Common Features: Early Antibiotic[f]	Common Features: Multiple Antibiotic[g]	Ref.
2004	Pro (PB)	4408	US	General	<1 yr	≥1 yr ≤5 yr	MR	MR	asthma 1–2 yr[k]: 1–2AB / >4AB; asthma 2–5 yr[k]: 1–2AB / >4AB	ND	ND	ND	*1.2 / *2.5 / 1.0 / 0.9	ND#	Y / N	55
2004	Retro (CSS)	1584 / 2539	New Zealand	"childhood infections" / "general population"	<1 yr	≤6-7 yr	PR	PR	groups combined due to similar prevalences	ND	*2.10	*1.52	*1.40	ND#	ND	60
2004	Retro (PB)	746	UK	General	<5 yr	adults	MR	PR (SPT)		1.01	ND	1.04	*1.09	ND#	ND	53
2005	Retro (PB)	26,400	Korea	General	<1 yr	≤7–12 yr	PR	PR		ND	ND	ND	*1.86	ND#	Y	54
2005	Pro (PB-BC)	448	US	General	<6 mo	6–7 yr	MR	SPT		1.48	ND	ND	ND	N	ND	59

*A significant association as defined by a reported 95% confidence interval for the given odds ratio that spans values >1.0. [a]Retro = retrospective; Pro = prospective; PB = population based; BC = birth cohort; CSS = cross-sectional study; FH = family history (inclusion requirement); C-C = case-control. [b]Evaluation critieria based on subject age at antibiotic treatment and allergy diagnosis; for allergy diagnosis, ≤ signifies allergy "ever" diagnosed up to age at study conclusion. [c]MR = medical record; PR = patient recall (typically by parent or guardian); SPT = skin-prick test (used to diagnose atopy). [d]Study population stratified based on criteria listed in this column; Note - when population stratified into more than 2 groups based on antibiotic courses only the first and last groups are included here. [e]Adjusted odds ratios reported for developing allergic disease in the population with antibiotic use compared to reference population with no antibiotic use (unless otherwise noted); Dermatitis = atopic dermatitis or eczema; Rhinitis = allergic rhinitis or hay fever; ND = not determined in the study. [f]Did the authors note increasing odds ratios with earlier antibiotic treatment? Y = yes; N = no; ND# = not determined but only early antibiotic treatment evaluated in the study. [g]Did the authors not increasing odds ratios with increasing number of antibiotic courses? Y = yes; N = no; ND = not determined in the study. [h]Only unadjusted odds ratios reported for dermatitis, rhinitis, and asthma. Students recruited from Rudolf Steiner (RS) schools and the information about characteristic features of the anthroposophic lifestyle were collected for subjects. [i]Odds ratio for ≥2 antibiotic courses group versus reference group of ≤1 antibiotic courses reported. [k]Groups stratified based on age at the time of asthma diagnosis (1–2 yr or 2–5 yr).

possible to draw the conclusion from these epidemiological studies that antibiotic use in and of itself contributes to development of allergic disease, because other interpretations for these data exist. In particular, early symptoms of allergy/asthma may be misdiagnosed and "treated" with antibiotics. For this reason, authors adjusted odds ratios for potential confounding factors oftentimes, including physician consultation behavior and respiratory infection history. In general population-based studies with a cumulative total of more than 65,000 subjects from Europe, the United States and Korea, six different groups reported a significant increase in the odds ratio for developing asthma in the subjects treated with antibiotics early in life.[49-54] Another study found that antibiotic use in the first year of life was associated with asthma diagnosed in the second year but not in years 2-5, leading the authors to conclude that antibiotic use did not contribute to asthma development but that antibiotic use was more common in asthmatic children.[55] No significant association was reported between antiobiotics and asthma in a different study, but this group used subjects that received less than or equal to one course of antibiotic treatment as the reference while all other studies used subjects that received none.[56] A number of reports highlighted associations between an increase in the odds ratio for asthma and a greater number of antibiotic courses and/or earlier antibiotic treatments.[49-52,54,55,57] A lack of association between antibiotic use and atopy in five studies that used skin-prick tests to monitor atopic sensitization does question the manner in which antibiotic use increases the odds of subsequent asthma development.[50,51,53,58,59] Still, significant associations with atopic dermatitis (eczema) and/or allergic rhinitis (hayfever) were made as often, as they were not.[49-53,57,58,60-62] We hypothesize that antibiotic use may be one factor of the westernized lifestyle that contributes to allergies in susceptible individuals through it's effects on the microbiota, but more studies on those with a predisposition to allergy are needed. In one study, antibiotic use was associated with hay fever development in children with a family history of allergies, but this was not the case in two other studies.[58,61,62] Still, given these data and those regarding the association between allergies and the microflora composition, it is plausible that antibiotic use does promote the manifestation of allergic disease in susceptible individuals by altering the microflora. This is a testable hypothesis awaiting further basic investigation in animal models.

Some early experiments on the rodent microbiota demonstrated that it changed rapidly upon altering the diet (for a review see ref. 65).[63,64] Perhaps even more relevant to the current health issues were later studies demonstrating that rodents fed an enriched bread diet exhibited a significantly delayed recovery of the microbiota ratios following antibiotic treatment compared to rodents fed a standard diet.[64] The role of diet in increasing or decreasing the incidence of allergic airway disease has been noted in a number of studies.[66-70] While antibiotic use in the Mediterranean countries of Spain, France, Italy and Greece is not necessarily different than that in the UK, Ireland or Australia, the asthma rates noted in the 1998 ISAAC report indicated that the incidence of asthma in these "Mediterranean Diet" countries is significantly lower.[8] Significant attention has been paid to the role of dietary metabolites in direct immune system interactions during allergic responses, but the diet also has a significant affect on the composition of the microbiota.[66,67]

The role of fatty acids in allergic airway disease is not understood. There is a rough association between national polyunsaturated vegetable oil consumption and corresponding national incidence of atopy and asthma.[68] Another study of ten European countries investigated the association between dietary trans-fatty acids and the prevalence of childhood asthma and allergies. There was a positive association between dietary trans fatty acids (expressed as percent of energy intake) and the prevalence of asthma, allergic rhinoconjunctivitis and atopic eczema.[70] Another example is a study of dietary fat intake vs. asthma in 478 men, 68 yrs. of age, who were randomly selected from all the men born in Malmo, Sweden in 1914. The study concluded that men with asthma had a significantly higher intake of fat than men without asthma.[71] Generally speaking, these studies and others, discuss the possible role of dietary fats as substrates and modulators of leukotriene and prostaglandin production that would, in turn, augment allergic responses.

While dietary fatty acids may directly modify host responses, dietary fatty acid intake also plays a significant role in shaping the population dynamics of the microbiota. For example, a number of strictly *anaerobic bacteria* have strict requirements for long-chain fatty acids.[72] Thus, changes

in dietary fats can alter one or more species of GI microbes, which in turn, can alter the numbers of other species of microbes by altering competitive exclusion dynamics. However, the argument continues to be circular in that the GI microbiota also plays a significant role in the metabolism of lipids and sterols, including biohydrogenation of sterols and fatty acids (for reviews see refs. 73-75). In the end, there is a tight relationship between dietary fat intake and modulation of GI microbiota dynamics. This raises the question of whether an alteration of GI microbiota populations by dietary fats is an underlying component of the dietary fat-asthma association.

An association has also been noted between higher dietary antioxidant intake and lower incidence of asthma (for a review see ref. 66). One class of antioxidant compounds includes polyphenols, which are found in high concentration in the skin of raw fruits and vegetables. A study in Italy demonstrated a correlation between high vegetable consumption and lower incidence of asthma.[69] Other studies have demonstrated an association between low fruit and vitamin C consumption and impaired lung function.[66] When antioxidant supplementation was examined as a preventative therapy prenatally, differential results were observed. In atopic women, vitamin E supplementation was negatively associated with atopic disease in infants, while vitamin C was positively associated with atopy.[76] However, a separate study found that only vitamin C consumed as part of the diet (as opposed to a supplement) ended up in breast milk. In this study, results demonstrated that increased vitamin C in breast milk was associated with a reduced risk of atopy in the infant. It was noted almost a century ago and confirmed in numerous other studies that there are significant differences in the GI microbiota between breast-fed and bottle-fed infants.[34,65] The chief difference between these two feeding regimens is that the microbiota of breast-fed infants is composed mainly of *lactic acid bacteria*, while the microbiota of bottle-fed infants is more diverse, composed of a mixture of *anaerobic bacteria* as well as aerobic species.[34] Thus, the role of breastfeeding in protecting against atopic disease may also be related to the beneficial effects on the microbiota.

A very interesting examination of the role of the westernized lifestyle in promoting allergic disease is found in studies on individuals who live in westernized communities and have adopted an anthroposophic lifestyle. Those leading an anthroposophic lifestyle restrict the use of antibiotics, pyretics and vaccinations and ingest fermented foods containing probiotic organisms such as *lactobacilli*. Studies on this population of individuals also noted a decreased incidence of atopy compared to the surrounding community and fecal samples contained higher levels of *lactic acid bacteria*. One study demonstrated that children of families with an anthroposophic lifestyle had a decreased prevalence of atopy compared to children in neighboring areas.[77] In addition, fecal samples contained higher levels of *lactic acid bacteria*. There is a correlation between the number of characteristic features of an anthroposophic lifestyle and decreasing risk of developing allergies. Several features of the anthroposophic lifestyle are likely involved in promoting decreased rates of atopy.[78] However, a study investigating anthroposophic children revealed that the use of antibiotics early in life was significantly associated with development of asthma.[57] Furthermore, the number of courses of antibiotics during the first year was also associated with increased odd ratios for asthma. This indicates that antibiotic use within a cohort of children with similar lifestyles predisposes towards atopic disease. The study of anthroposophic individuals living in westernized communities represents a unique opportunity to study the westernized lifestyle apart from other environmental factors in promoting allergy.

Regulation of Mucosal Tolerance

The mucosal immune system monitors the epithelium of the respiratory, gastrointestinal and genitourinary tracts, where vital interactions with the outside world are undertaken. The mucosal immune is charged with guarding the epithelial surfaces to protect the host against infection, but inappropriate inflammation can damage the epithelium and impair important physiologic functions. For this reason, tolerance and inflammation are tightly controlled by complex, multi-layered regulatory mechanisms along the mucosa. Many features of the mucosal immune system commensurate with this task have been identified, including unique epithelial and innate and adaptive immune cells. Similarities in the structure and function of different mucosa-associated

lymphoid tissues (MALT) have encouraged the concept of a common mucosal immune system (CMIS), but differences do exist. A shared property central to the "Microflora Hypothesis" is the propensity to generate systemic tolerance to antigens encountered via the oral, nasal and airway routes.[79-82] Furthermore, the capacity to develop oral tolerance is dependent on the presence of the microbiota.[83] Passive tolerance, the act of not generating pro-inflammatory signals, is maintained as a result of constant interaction with the microbiota. Active tolerance involves suppression of inflammatory reactions and is the function of regulatory T-cell (Treg) populations. The question as to how the composition of the microbiota may affect the outcome of tolerance or allergy at distant sites is considered in this section.

MALT is organized into unique inductive and effector sites.[84,85] Inductive sites include specialized lymphoid follicles underlying the epithelium, such as nasopharynx-associated lymphoid tissue (NALT) in the upper airway and Peyer's patches (PP) and isolated lymph follicles (ILF) in the gut and downstream lymph nodes, including cervical and mediastinal LN draining the respiratory mucosa and mesenteric LN draining the intestinal mucosa. MALT effector sites include the epithelium and lamina propria. Studies of the organogenesis of secondary lymphoid structures indicate that PP, NALT and peripheral LN all have different requirements for developmental signaling by the lymphotoxin family, tumor necrosis factor family, or IL-7 pathways.[84] However, interactions with the microbiota are necessary for the generation of normal MALT. Germ-free animals have hypoplastic PP with few germinal centers (GC).[86,87] Unlike PP whose organogenesis is initiated in utero, NALT and ILF organogenesis is initiated after birth in response to stimulatory signals provided in part by the microbiota.[88,89]

Antigens are acquired by LP-resident dendritic cells (DC) directly by sampling the lumen, or indirectly through the action of specialized Microfold (M) cells situated in the epithelium which transfer luminal antigens to DC in underlying lymphoid tissues including NALT and PP.[90,91] Interactions between antigen-loaded DCs and CD4+ T-cells in the inductive sites determine the nature of the ensuing response. In particular, the activation state of the DC affects the outcome of tolerance or inflammation.[92] Evolutionarily conserved microbial products, termed pathogen-associated molecular patterns (PAMP), signal through pattern recognition receptors (PRR) that are highly expressed by DC. Signals received through PRR, including toll-like receptors (TLR), are synthesized in complex fashion that depends on the variety and duration of the stimulation.[93] Mice lacking functional TLR4 were susceptible to food allergy induction whereas wild-type controls were not.[94] The function of TLR4 in maintaining tolerance relied on the bacterial microflora because antibiotic-treated, wild-type mice exhibited sensitivity unless the flora was allowed to repopulate. While an in depth discussion of PRR signaling is beyond the scope of this chapter, it is further noted that (1) signaling through TLR9 on DC induced Treg activity in one study and (2) TLR4 or TLR9 signaling to DC abolished Treg-mediated suppression in another.[95,96] Therefore, interactions with microbial products can influence the propensity for DC to stimulate tolerance or inflammation.

Activated T-and B-cells travel through the lymphatics and eventually the thoracic duct where they enter the bloodstream and traffic to effector sites in the lamina propria (LP) and epithelium. It is suggested that lymphocytes return to the tissue in which they were activated and PP express mucosal vascular addressin cell-adhesion molecule 1 (MADCAM1) whereas NALT express peripheral-node addressin.[97] However, lymphocyte migration to MLN relied on adhesion molecules that bind both mucosal and peripheral node addressins, indicating that MLN could serve an important role as a crossover point for cells activated in the GALT. Activated B-cells undergo immunoglobulin class switch to IgA in PP and NALT and are chemotactically attracted to the epithelium where they differentiate further into antibody-secreting plasma cells.[98-100] Mice deficient in activation-induced cytidine deaminase cannot class switch to IgA and are defective in somatic hypermutation of immunoglobulin genes. As a result, there is a 100-fold expansion in anaerobic bacteria in the small intestine, illustrating the critical role of mucosal immunoglobulin in regulating the microbiota.[101] Likewise, "effector memory" CD4+ and CD8+ T-cells and presumably, Treg also take up residence in the LP.[102,103]

Antigens encountered at mucosal sites preferentially lead to tolerance induction. As mentioned above, antigens delivered via oral, nasal, or airway routes can induce systemic unresponsiveness to the particular antigen. Oral tolerance has been rigorously investigated for its therapeutic potential in the setting of autoimmune diseases, where prevention or amelioration was seen in mouse models of rheumatoid arthritis, multiple sclerosis and type I diabetes.[104-106] Oral tolerance can also inhibit the cardinal features of allergic airway disease in the well-studied OVA model.[107] In human adult volunteers, oral tolerance was demonstrated after a prolonged feeding regimen with keyhole limpet hemocyanin resulted in T-cell, but not B-cell, systemic unresponsiveness.[108]

Shortly after intranasal inoculation, fluids, particles and microbes introduced into the nasal cavity are largely found in the GI tract.[109-111] In mice, intranasal inoculation of a volume as small as 2.5 μl still largely ends up in the GI tract.[110] Thus, inhaled micro-particulates (which comprise the vast majority of aeroallergens) are also swallowed and could potentially induce tolerance via the oral tolerance mechanism. Studies have demonstrated that (1) the normal microbiota is required for the generation of oral tolerance since it cannot be generated in germfree mice and (2) conventionalization of germfree mice with normal microbiota can restore the ability to generate oral tolerance in these mice, indicating that tolerance continues to be regulated by the microbiota long after the post-natal period.[112,113] Therefore, dynamic interactions between the immune system and the microbiota are necessary to promote the induction of tolerance to inhaled and ingested antigens.

The mechanisms mediating oral tolerance depend on the dose of antigen administered.[114,115] High doses lead to anergy/deletion, but for this discussion the ability of low dose antigen to induce suppression is most interesting. The results of depletion, reconstitution and adoptive transfer studies convincingly demonstrate that tolerance in this setting is mediated by CD4+ regulatory T-cells (Treg).[116-119] The mechanisms of Treg-mediated suppression are not entirely known, but it is clear that Tregs require T-cell receptor stimulation and that production of immunosuppressive cytokines, IL-10 and TGFβ, are critical mediators in vivo.[120] Thus, Tregs require specific activation but can mediate nonspecific suppression in what is termed "bystander suppression." As mentioned previously, DC-T-cell interactions control the immunological outcome and expansion of the DC population with the in vivo administration of flt3 ligand can enhance the induction of oral tolerance.[121] Similarly, repeated antigen exposure in the airways leads to the development of dominant tolerance mediated by CD4+ Treg.[122] Depletion and adoptive transfer studies of lung DC, indicate that these cells are crucial to tolerance induction at this mucosal site as well.[123,124] In humans, genetic deficiency in the FOXP3 gene that controls the transcriptional program for Treg commitment leads to a complex syndrome characterized by severe autoimmune and allergic manifestations.[125-127] Moreover, defects in the ability of Treg from allergic patients to inhibit allergen-specific Th2 responses support the functional role of these cells in maintaining tolerance.[128-130] It should be clear that the inhibitory DC-Treg axis is core to mucosal tolerance and hence, the "Microflora Hypothesis."

Experimental Evidence that Altered Microbiota Can Promote the Development of Allergic Airway Disease

The yeast *C. albicans* is the major fungal species in the human microbiota. It resides in low numbers on almost all mucosal surfaces (for a review see ref. 131) and its numbers can increase following disruption of the microbiota (diet, antibiotics) or by specific changes in other host defense mechanisms (physical barrier, innate immunity and adaptive immunity). In humans, yeast infections of mucosal sites are one of the most common side effects of antibiotic therapy.[39,47,132-134] The ability of the bacterial microbiota to control or prevent *C. albicans* colonization is due to both competitive exclusion of favored niches and by production of growth-altering metabolites such as short chain fatty acids (SCFA) (for a review see 18).[135-139] SCFA, such as butyric acid, are by-products of anaerobic fermentation by the normal probiotic members of the microbiota and also possess anti-inflammatory activity (for a review see 144).[140-143] We have recently demonstrated that a number of probiotic *Lactobacillus* strains can inhibit *C. albicans* hyphal transformation,

which is a key step for epithelial invasion and commensal-to-pathogen switch.[145] Thus, control of *C. albicans* by the normal microbiota (especially the probiotic species) is very important.

Our laboratory has recently developed a mouse model of antibiotic-induced GI microbiota disruption that is accompanied by stable increases in gastrointestinal enteric bacteria and *C. albicans* levels.[19,146] Using this model, we have addressed whether microbiota disruption can promote the development of an allergic airway response to mold spore (*A. fumigatus*) or ovalbumin challenge (Fig. 1). These studies utilized immunocompetent mice and did not involve previous systemic antigen priming, typically used for breaking airway tolerance to these allergens, but instead explored sensitization after exposure at a natural site. There was also no evidence of microbial growth in the lungs or inflammation in the GI tract in this model. The parameters measured included pulmonary eosinophilia, total serum IgE, lung leukocyte IL-5, IL-13 and IFN-γ and goblet cell metaplasia. All of these parameters were significantly elevated in the microbiota-disrupted mice. Mice with unaltered microbiota did not develop an allergic response following intranasal challenge with either mold spores or ovalbumin. The response did not develop in IL-13 deficient mice or mice that had been depleted of CD4 T-cells. In addition, vigorous allergic airway responses could be generated in both C57BL/6 and Balb/C mice following microbiota disruption and antigen challenge but not in antigen-challenged "normal microbiota" C57BL/6 and Balb/c mice. The presence of *C. albicans* in the GI tract was required to break airway tolerance. Thus, these studies demonstrate experimentally that antibiotic treatment, including fungal microbiota growth, can break airway tolerance to an aeroallergen such as mold spores or an experimental nonfungal allergen such as ovalbumin.

Rook and Brunet have proposed that interactions with certain microbes ("Old Friends") are wired into existing immunoregulatory networks based on their constant presence in our environment during our evolutionary past.[16] In a series of studies, they demonstrate inhibition of allergic airway disease by pretreatment with heat-killed *Mycobacterium vaccae*, a ubiquitous saprophytic mycobacterium, either by subcutaneous or, most importantly for this discussion, by intragastric administration.[147-149] Moreover, treatment prevented Th2 sensitization through stimulation of inhibitory DC and Treg and not through a Th1 response. In another study, oral administration of oligodeoxynucleotides containing bacterial CpG motifs, TLR9 ligands, inhibited some parameters of allergic airway disease in the OVA model.[150] Respiratory exposure to the TLR4 ligand, LPS, can augment allergic responses in a rather complex pattern that is at least in part dose dependent.[151-153]

Figure 1, viewed on following page. Experimental evidence that altered microbiota can promote the development of allergic airway disease. The effect of experimental microbiota disruption (MBD), consisting of 5 days administration of the antiobiotic cefoperazone in the drinking water (0.5 g/ml) immediately followed by a single oral gavage of live *Candida albicans* strain CHN1 (10^7 CFU), was evaluated on the subsequent response to aeroallergen exposure in two separate mouse models. In the first, C57BL/6 unmanipulated control mice (–MBD) and altered microbiota mice (+MBD) were challenged intranasally with viable spores of the fungus *Aspergillus fumigatus* (10^7) on days +2 and +9 after MBD and mice were evaluated 72 hr after the final dose. In the second model, BALB/c mice –/+ MBD were challenged intranasally with the model allergen OVA (50 ug) on days +2, 5, 9, 12, 16 and +19 after MBD and mice were evaluated 48 hr after the final dose. A) Low-power magnification of H&E-stained lung sections depicts the extent of inflammation. B) High-power magnification highlights the presence of eosinophils in +MBD groups. C) Serum total IgE was measured by ELISA. D) The total number of lung eosinophils was the product of the % eosinophils within the lung leukocyte population as determined by routine differential count and the total number of leukocytes present in the lung tissue recovered by a process of mechanical disruption, enzymatic digestion and leukocyte enrichment by Percoll gradient centrifugation. E) The supernatant from a 24 hr culture of freshly isolated lung leukocytes (5×10^6 cells/ml) was analyzed for IL-13 by ELISA. For all graphs shown there was a significant difference in the –MBD and +MDB groups as determined by a two-tailed t test ($p < 0.05$) with the exception of IL-13 in BALB/c + OVA experiment ($p = 0.0557$).

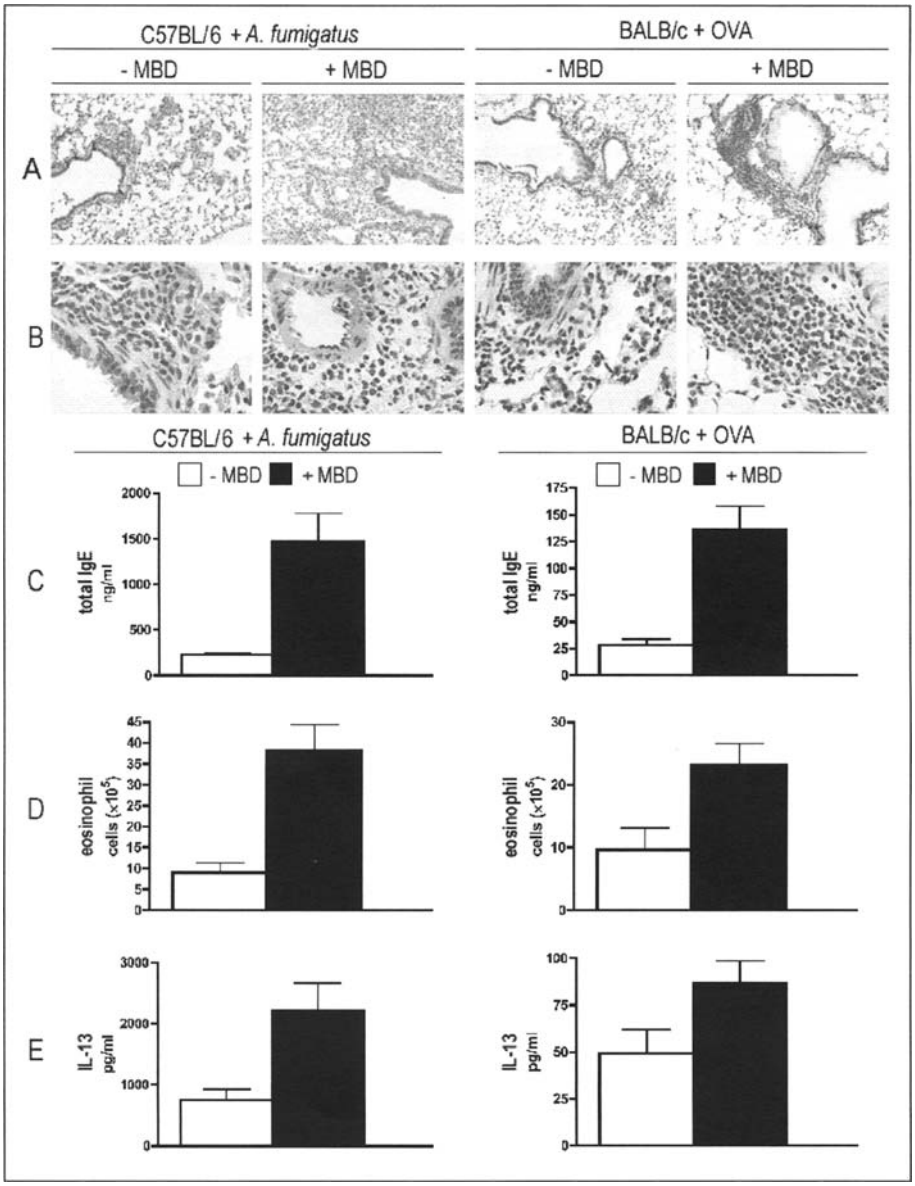

Figure 1, legend viewed on previous page.

The ability of certain microbial products to induce immunogical tolerance to allergens has lead to clinical testing for the treatment of allergic rhinitis and asthma.[154]

Summary

Currently, the immune mechanisms by which the microbiota may influence the manifestation of allergy in susceptible individuals are not fully elucidated (Fig. 2). It has been shown that the microbiota of allergic individuals differs from that of non-allergic ones and that differences are

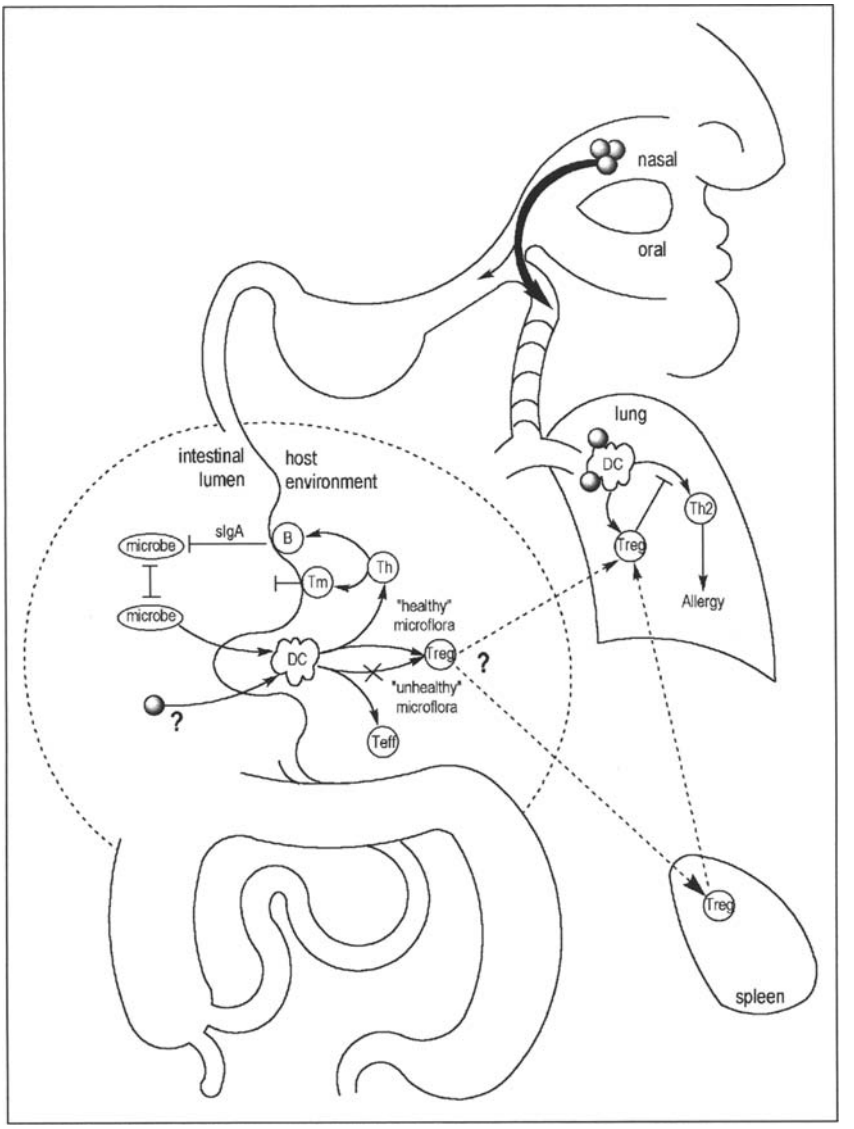

Figure 2. Possible gastrointestinal microbiota-dependent regulatory T-cell-mediated control of allergic airway responses. This line drawing depicts several events that may be involved in the microbiota-dependent regulation of allergic responses in the airway. "Healthy" microflora promotes proper immunoregulation and tolerance that is maintained by tolerigenic dendritic cells (DC) and regulatory T-cells (Treg). Under these circumstances, effector sites underlying the epithelium are populated by plasma cells (B) producing sIgA and various leukocytes, including memory CD4+ and CD8+ T-cells (Tm), poised to respond to pathogenic organisms. "Unhealthy" microflora interferes with proper immunoregulation and tolerance at distant sites including the lung. It is possible the Treg, which are known to inhibit Th2-mediated allergic airway disease, are altered in this setting. It is not known if inhaled allergens, which are also swallowed, result in the generation of antigen-specific Treg in the gut, or how Treg, dependent on the GI microbiota for generation or function, may eventually effect the outcome of exposure to allergens in the lung.

detectable before the onset of atopy, consistent with a possible causative role. Other features of the westernized lifestyle that are known to alter the microbiota, such as antibiotics and diet, are also associated with allergy in humans. Allergies result from a breakdown in tolerance, which is the typical, healthy response to nonthreatening materials encountered at mucosal sites. Dendritic cells are key in the development of tolerance or immunity/allergy and their propensity to develop one or the other response depends in large part on signals they receive through PRR from microbial products. DC in the GALT are refractory to many PRR stimuli due to constant exposure, but alterations in the microflora can increase their willingness to respond. In many if not all situations, tolerance is mediated by CD4+ Tregs. Tolerance induced via mucosal administration is routinely manifested at distant sites indicating some form of co-ordinated regulation. Inhaled antigens are also swallowed, suggesting the possibility of a shared mechanism for the induction of antigen-specific oral and respiratory tolerance. Also, Treg induce non-antigen-specific "bystander suppression" due to the nonspecific action of various mediators, like IL-10 and TGF-β. In this way, tolerance could be maintained throughout the common mucosal immune system through local spreading, or "infectious tolerance." In that scenario, innate cells, including DC, could maintain a quiescent mucosal immune system under physiologic conditions, but induce inflammation in response to activation signals through short-circuiting of the Treg network. Signals from the microbiota feed forward through antigen presentation and T-helper activity to the level of sIgA, which in turn regulates the microbiota. Therefore, alterations in the microbiota, resulting from antibiotic use for instance, could introduce a lot of "noise" into the system by perturbing innate and eventually downstream adaptive immune responses that maintain homeostasis between the host and the microbiota that may take a while to "quiet down". Evidence discussed in this chapter indicates that this situation may pose a risk for the development of allergy. This could occur in an antigen-specific manner if, for instance, inhaled particles induce antigen-specific tolerance upon reaching the gut that is critical in preventing allergic responses to subsequent encounters in the airways. Alternatively, or perhaps additionally, non-antigen-specific control of systemic immune tolerance may depend on "healthy" interactions with the GI microbiota that induce a complex series of immune responses that ultimately lead to proper immunoregulation, tolerance, of the response to nonpathogenic foreign materials encountered at the many sites where interactions with the outside world are a feature of life.

Future Perspectives

It is clear that predisposition to allergic disease is a complex function of an individual's genetic background. As is the case with multi-gene traits, environmental factors have important phenotypic consequences. Information on the etiologies of allergic diseases benefit from studies on genetic polymorphisms and environmental exposures that are associated with allergic disease in human populations. The link between the microbiota composition and allergies is very intriguing. A great effort is required to construct definitions of "healthy" and "unhealthy" microbiota in human populations associated with tolerance and allergy, respectively. However, this type of evidence cannot prove that a particular combination of microbiota constituents can cause allergic disease. Thus, generating direct proof for the "Microflora Hypothesis" of allergic disease will rely largely upon experimental animal models and well-controlled human intervention studies such as are now being proposed and carried out with probiotic therapies in children. The accumulating evidence also suggests that the medical establishment should more seriously consider the role of diet in chronic disease, think seriously about prescribing long-term antibiotics for nonlife threatening conditions and also consider probiotic and prebiotic strategies for patients coming off of antibiotic therapy.

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CHAPTER 11

The Damage-Response Framework of Microbial Pathogenesis and Infectious Diseases

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Abstract

Historical and most currently held views of microbial pathogenesis and virulence are plagued by confusing and imprecise terminology and definitions that require revision and exceptions to accommodate new basic science and clinical information about microbes and infectious diseases. These views are also inherently unable to account for the ability of some microbes to cause disease in certain, but not other hosts, because they are grounded in singular, either microbe-or host-centric views. The damage-response framework is an integrated theory of microbial pathogenesis that puts forth the view that microbial pathogenesis reflects the outcome of an interaction between a host and a microbe, with each entity contributing to the nature of the outcome, which in turn depends on the amount of host damage that results from the host-microbe interaction. This view is able to accommodate new information and explain why infection with the same microbe can have different outcomes in different hosts. This chapter describes the origins and conceptual underpinnings of and the outcomes of infection put forth in, the damage-response framework.

Introduction to the Damage-Response Framework

The damage-response framework is a theory of microbial pathogenesis that was first proposed in 1999 in an effort to account for the contribution of both the host and the microbe in microbial virulence and pathogenicity.[1] Until that time concepts of microbial pathogenesis were largely microbe-or host-centric, in that they attempted to explain microbial virulence in the context of microbial properties or host susceptibility, respectively. Microbe-centric views regard virulence and pathogenicity as singular microbial traits, e.g., as the result of the action of a microbial factor or determinant that injures the host. Host-centric views regard virulence and pathogenicity as host-dependent outcomes that result from a defect or deficiency in the host. In contrast, the damage-response framework is neither microbe-nor host-centric but focuses on the outcome of the host-microbe interaction and emphasizes that host damage is the common denominator that is relevant to any host-microbe interaction. The damage-response framework reconciles microbe-and host-centric views by incorporating the recognition that both the microbe and the host contribute to pathogenicity and virulence. It is based on three tenets that are considered to be both obvious and incontrovertible: (1) that microbial pathogenesis requires two entities, a host and a microbe

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and that the two entities must interact; (2) that the host relevant outcome of host-microbe interaction is damage to the host; (3) host damage can occur as a result of microbial factors, host factors, or both.[2] These tenets are represented graphically by the basic damage-response curve, a U shaped curve that depicts host damage on the Y axis as a function of the host immune response, which is depicted from weak to strong along the X axis. The U shape of this curve illustrates that host damage can be maximal in the setting of a weak or a strong host response (Fig. 1).

Conceptual Origin of the Damage-Response Framework

The damage-response framework originated as a teaching tool in the graduate microbial pathogenesis course at the Albert Einstein College of Medicine in the mid-1990s. While teaching we found it very difficult to convey to students the concept that some microbes were pathogenic only in certain hosts using the then existing treatises on pathogenicity and virulence. The inability of either microbe-centric or host-centric views to account for the late 20th century emergence of diseases caused by microbes previously considered to be nonpathogens and the emergence of the diseases caused by these microbes in individuals with immune impairment was the catalyst for proposing a different approach to the problem.[3] These microbes included *Candida albicans* and *Staphylococcus epidermidis*, which emerged as leading causes of bloodstream infections when they had long been held to be nonpathogens.[4,5] The late 20th century witnessed an unprecedented increase in individuals with immune impairment due to predominantly 4 factors: (1) the use of plastic catheters to deliver intravenous fluids and medications in the hospital setting; (2) the rise in antibiotic use, overuse and misuse;[6] (3) the development and use of immunosuppressive therapies for malignancy and to combat organ rejection in the setting of organ transplantation; and (4) the HIV/AIDS pandemic (Fig. 2). Each of these factors led to the emergence of distinct populations of individuals with impaired immunity and it was among these individuals that many microbes previously considered to be nonpathogens were associated with disease. The observation that microbes previously considered to be nonpathogenic could be pathogens led to the concept of microbial 'opportunism',[7] an unfortunate term that introduced the anthropomorphic view that these microbes were somehow taking advantage of the host to cause disease. In fact, many of the pathogens labeled as opportunistic were components of the normal microbial flora, such as *Candida albicans* and *Staphylococcus epidermidis*. The convergent emergence of diseases caused by microbes long held to be nonpathogens and newly emergent populations of immunocompromised individuals brought to the fore that infectious diseases can only occur in susceptible individuals. Although the veracity of this statement is immediately obvious, this notion is distinctly absent in microbe-centric views which regard microbial virulence as a microbial property. The veracity of this statement is further underscored by the fact that diseases caused by vaccine-preventable microbes, eg smallpox, do not occur in immune individuals and that the clinical manifestations of infectious diseases often reflect the host inflammatory response, in some cases, even in the absence of the causative microbe. These points, which are largely agreed upon by the infectious diseases and microbial pathogenesis fields, issue a serious challenge to prevailing definitions of pathogenicity and virulence, since the same microbe could be either a pathogen or nonpathogen, depending on the host.

The Lexicon of the Damage-Response Framework

A central feature of the Damage-response framework is a simple, self explanatory lexicon that does not require exceptions or corollaries to define the components of microbial pathogenesis and virulence. The key to understanding the lexicon is that according to the damage-response framework, the essential components of microbial pathogenesis reduce to two entities, hosts and microbes and the damage that occurs in the host as a result of their interaction. Furthermore, the outcome of the interaction can change as a function of time depending on the amount of damage that occurs in the host. The damage-response framework does not view pathogens and nonpathogens as intrinsically different; based on incontrovertible evidence that the same microbe can be a pathogen or nonpathogen, depending on the host. Hence, the terms 'pathogen' and 'nonpathogen' only have

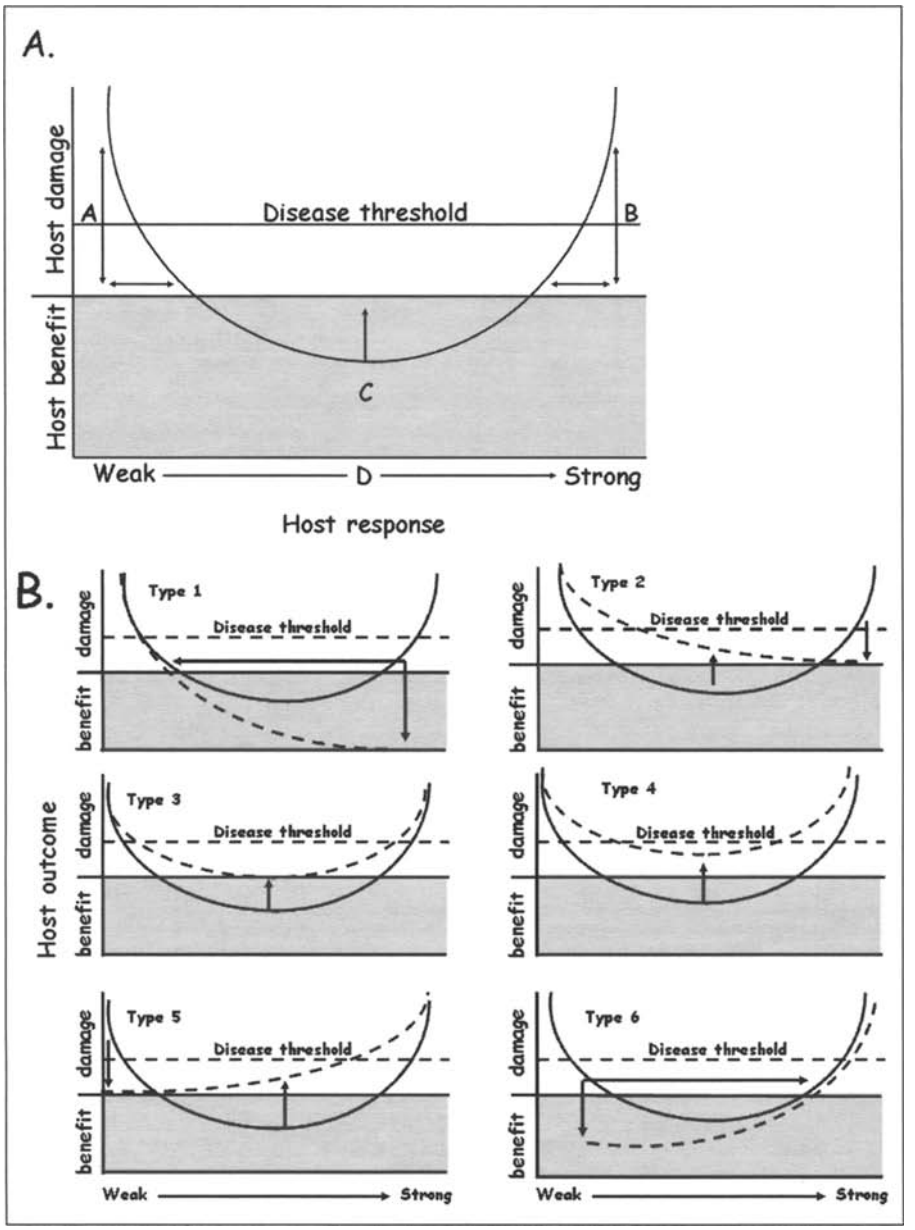

Figure 1. The damage response curve. Host damage is depicted as a function of the host response along a continuum from weak to strong. A) The solid U shaped curve demonstrates that certain host- microbe interactions confer a host benefit. The arrow (C) illustrates that the curve can shift upwards. The arrows at each side of the curve (A, B) illustrate that the curve can shift downward and to the left and right. B) Damage-response curves that reflect the outcome of different host- microbe interaction can be derived from the basic curve. Examples of microbes that result in these types of curves are as follows: Type 1—*Staphylococcus epidermidis*; Type 2—Hepatitis A virus; Type 3—*Aspergillus spp.*; Type 4—*Histoplasma capsulatum*; Type 5—SARS coronavirus; Type 6—*Helicobacter pylori*.

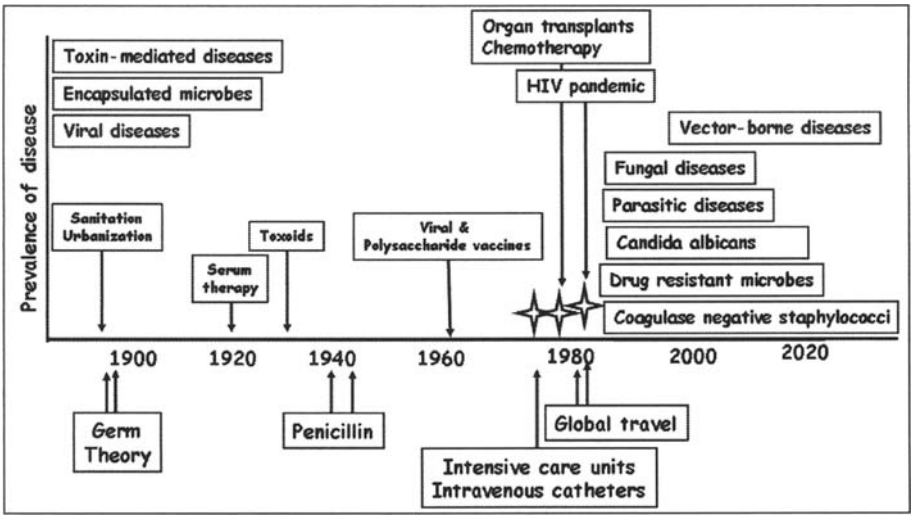

Figure 2. The spectrum of infectious diseases 1900-2000. The prevalence of disease due to the indicated microbes is shown on the Y axis as a function of time and the innovations and factors depicted on the X axis.

meaning in the context of a given host. The damage-response framework defines a pathogen as a microbe with the potential to cause damage in a host.[8] This definition avoids linking the nature of a pathogen to mechanisms by which it causes disease and encompasses microbial diversity, which extends from microbes that invade host cells to those that do not, or are macroscopic, such as *Shigella sp* and *Vibrio cholera* and *Ascaris lumbroides*, respectively, to those that have a normal niche, such as *Candida albicans* and *Staphylococcus epidermidis*, to those that are encoded by the host, such as prions. Virulence is defined as the relative capacity of a microbe to cause damage in a host.[9] The term 'relative' is necessitated by the fact that, at present, damage cannot be fully quantified; because precise readouts of host damage remain limited and available tools and platforms are insufficient for quantification. Furthermore, virulence has been and continues to be a relative term since any measurement of virulence is relative to a control condition or strain. Despite this gap, there is little difficulty in identifying or agreeing upon currently available readouts of host damage. When host damage surpasses a threshold that maintains host homeostasis, clinical disease occurs. With these definitions, the Damage-response framework dispenses with imprecise and confusing terms, such as nonpathogen, partial pathogen, primary pathogen, opportunistic pathogen, commensal and saphyrophyte. The problems of imprecise and shifting terminology are immediately apparent when one considers a microbe such as *Candida albicans* which is considered a commensal in most hosts, an opportunistic pathogen in patients with impaired immunity and even a primary pathogen in women with no obvious immune deficit that suffer from candidal vaginitis. The damage-response framework defines the term infection as the acquisition of a microbe, rather than to describe an illness or condition. This enables a more precise understanding of microbial pathogenesis that is consistent with the fact that infection with a microbe is not synonymous with it causing damage or disease.

The Damage Response Curve

The U shaped damage-response curve illustrates the complex origins of host damage by depicting it on the Y axis as a function of the host response along a continuum from weak to strong on the X axis (Fig. 1). The curve is U shaped, because host damage can occur in the setting of either a weak or a strong host response. The host response encompasses the full range of host immunity such that

weak and strong responses lack essential components that are required for the normal, or appropriate, response, which results in a minimum amount of damage, most likely due to counterbalancing responses. For example, the response to a microbe often produces an initial inflammatory response, which is later counterbalanced by a dampening of the response. The absence of an appropriate initial or a counterbalancing response can each result in host damage. Damage in the setting of a weak host response often reflects microbe-mediated damage, such as that caused by the action of microbial factors and damage in the setting of a strong host response often reflects host-mediated damage, such as that caused by excessive inflammation. Microbial factors that cause host damage include capsular polysaccharides, toxins, proteases and components that are toxic to host cells. Most of these factors cause more damage in the setting of weak responses. Host factors that cause host damage include immune complexes, cytokines, chemokines and microbicidal peptides. The recognition that host damage can occur at the extremes of the host response underscores that the outcome of microbial infection is an interaction, whereby singular host responses are insufficient to prevent or minimize host damage and an interplay that achieves a balanced response is most successful at damage control. The damage-response curve is inherently flexible and can be used to plot any host-microbe interaction.

The States of Infection

In addition to depicting host damage as a function of the host response, the damage-response framework also depicts host damage as a function of time. Hence, damage is a function of the host response at a given time (Fig. 1) and damage is a function of time for a given host response (Figs. 3, 4). According to this schema, there are 5 outcomes of microbial infection: elimination; colonization, commensalism, disease and latency (Fig. 3).[3,10] Colonization, commensalism, disease and latency are distinguishable by the amount of host damage over time. Changes between these states occur, usually as a result of a change in the host immune response (Figs. 3, 4).

Colonization is a state in which the amount of host damage is potentially measurable, but less than the disease threshold.[10] Although the methodology for measuring damage in states of colonization does not currently exist we note that this state is often associated with the development of an immune response which may reflect the occurrence of some degree of damage that is less than that which translates into disease. For most microbes, colonization is a transient state,

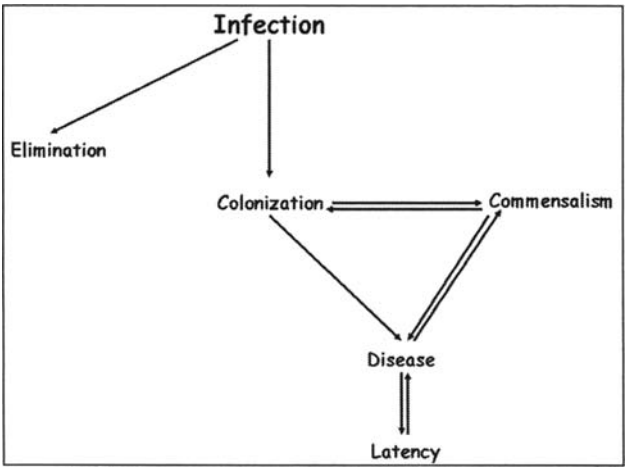

Figure 3. The five outcomes of microbial infection. The interrelationships between colonization, commensalism, disease and latency are depicted by arrows between the relevant outcomes. Factors that induce change from one state to another include immunosuppression, reduced barrier immunity (due to the insertion of vascular catheters), cytotoxic agents and therapy.

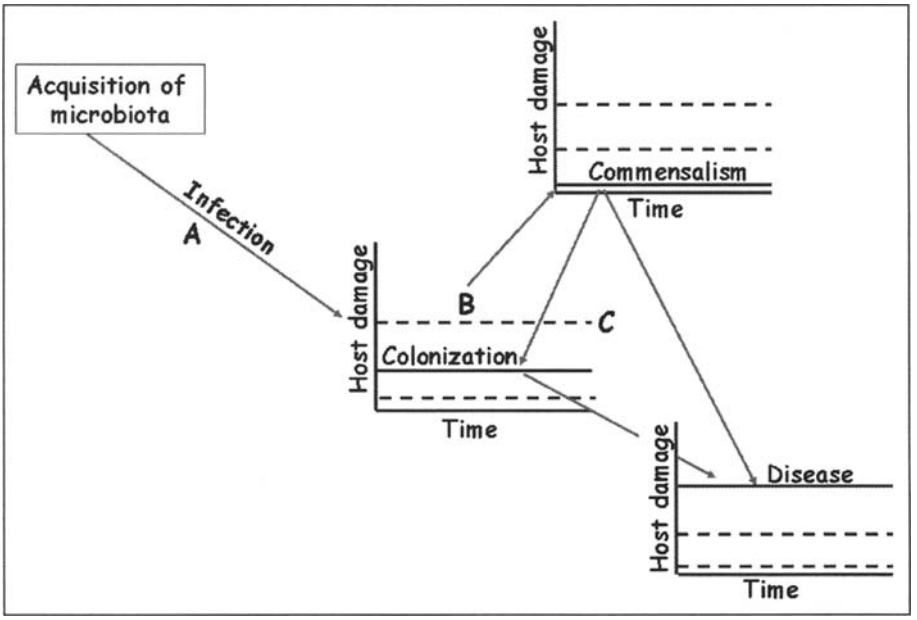

Figure 4. The acquisition of the microbiota and transitions between commensalism, colonization and disease. The relevant state is depicted by a solid line, the possible transition states are depicted by dashed lines. The transitions from infection to colonization (A) and from colonization to commensalism (B) occur early in life. The transition from commensalism to colonization or to disease, directly or indirectly, can occur when the microbiota is disrupted or eliminated due to invasion of skin or mucosal surfaces with catheters or surgery, antibiotic or cytotoxic therapy, or immunosuppression, or when host factors compromise its functioning (C).

during which the microbe can be isolated from the host and may be evidence of a host immune response. The types of host responses or damage that accompany colonization include serological evidence of infection, cellular responses that result in tissue responses, such as granulomas or giant cells and immune responses that result in inflammation and cellular recruitment. Whether the stimulus for such immune responses is microbe-mediated damage is uncertain at this time. The state of colonization can lead to elimination or transition to commensalism or disease. Elimination can result from immune mechanisms, e.g., by an immune response to a respiratory microbe, such as *Streptococcus pneumoniae*, or intervention, such as antimicrobial agents. Colonization transitions to disease when the amount of damage exceeds the disease threshold. This occurs when host mechanisms or intervention fail to limit host damage. The failure of host mechanisms often reflects weak or inappropriate immune responses, such as those that predispose individuals with antibody and B-cell defects to disease with *Streptococcus pneumoniae*, or individuals with defects in cellular immunity to disease with *Cryptococcus neoformans*. Colonization changes to commensalism following microbial acquisition soon after birth.

Commensalism is a unique state in which host-microbe interaction that either provides a host benefit or no outcome, rather than resulting in host damage.[10] There is no host damage in the state of commensalism. The state of colonization becomes indistinguishable from commensalism when the amount of host damage attributable to colonization is negligible. Hence, *Staphylococcus aureus* in the nares of a chronic asymptomatic carrier may be indistinguishable from a commensal microbe, with the caveat that in aggregate, some of the microbes that assume the state of commensalism impart a host benefit. The host is generally defined as the entity that microbes inhabit. However, the number of microbes that inhabit the human body exceeds the number of human

cells, calling into question the definition of host. The gastrointestinal tract is inhabited by more than 10^{13} microbes, with more than 100 times the number of genes as the human genome.[11] Hence, the state of commensalism provides a host habitat for vast and complex microbial communities. These communities collectively referred to as the microbiota include microbes originally thought to be acquired soon after birth. However, an emerging body of evidence suggests that the human microbiota is even more diverse than previously suspected and influenced by a myriad of host factors.[12] The diversity amongst and the regulatory and immunomodulatory roles that the human microbiota play has only recently begun to be unraveled,[11] principally through the use of innovative techniques that allow for the identification of unculturable microbes.[13] In addition to unculturable microbes, scores of culturable Gram negative and Gram positive, anaerobic and aerobic bacteria and *Candida albicans* inhabit the human host. The acquisition of these microbes can be associated with damage and disease, such as necrotizing enterocolitis and disseminated candidiasis in infants. However, in most instances, the acquisition of these and other microbes is not associated with disease. Microbes that inhabit the gastrointestinal tract are thought to contribute to the development and maintenance of natural immunity.[14-16] Although the microbial determinants and mechanisms that stimulate immunity remain to be fully understood, the importance of the microbiota for normal immunity is supported by evidence that host damage ensues when there is a failure to acquire or disruption of the microbiota. When this occurs, there is a transition from the state of commensalism to the state of colonization or of disease. The microbiota can be disrupted by surgical intervention, antimicrobial therapy, cytotoxic agents and radiotherapy. In addition to contributing to natural immunity, the microbiota play an important role in maintaining the integrity of host tissues, through the elaboration of protective substances and via colonization resistance, including mechanisms resulting in inhibition of other microbes with a greater potential to induce damage from gaining access to host receptors and tissues.

Disease is a state where host damage exceeds the threshold for clinical symptoms. The state of disease can change to elimination with intervention or if host immune mechanisms are sufficient to reduce the amount of damage to below the disease threshold. An inability to reduce damage below the disease threshold can reflect a failure of host immune mechanisms or an intervention to eliminate a microbe or control damage, or both. Interventions for infectious diseases endeavor to treat or prevent the state of disease. Most available interventions focus on microbial elimination, but such therapies often do not control the host response, because the state of disease often reflects aspects of the host response that induce inflammation and enhance the inflammatory response. The state of disease can change to latency, a state in which the microbe remains in the host and vital, but induces damage that is below the disease threshold. The inability to reduce damage in the state of disease ultimately results in chronic disease or death.

Latency is a state that is characterized by a microbial presence, whereby survival of the microbe produces an amount of damage that is below the disease threshold.[10] Latency does not have an obvious host benefit although it is conceivable that changes to the immune system by continued stimulation with microbial antigens forestalls the development of other conditions, such as allergic diseases (e.g., 'hygiene hypothesis').[17] For example, helminth infections have been associated with protection against the development of asthma[18] and patients with positive tuberculin reactions indicative of latent *Mycobacterium tuberculosis* infection had reduced atopy.[19] Latency is a state in which a microbe survives in host cells in a manner that prevents it from elimination, often due to factors that allow it to escape host immune surveillance. Mechanisms that enable latency include the capacity for intracellular survival and persistence, such as for Herpes and other viruses, the induction of tissue responses that contain and control growth of the microbe, such as granulomas for Mycobacteria and fungi and residence in sequestered sites, such as for HIV. The state of latency can transition to disease with a change in the immune status of the host. Major risk factors for this transition are diseases and interventions that impair host immunity, such as immunosuppressive agents given for malignancy, inflammatory diseases and stem and organ transplantation and HIV.

In summary, the outcomes of host-microbe interaction result in 4 states, which differ only in the amount of host damage, or benefit. The states are not fixed by the microbe, but by the amount

of damage that ensues from a host-microbe relationship. Since the outcome of a host-microbe interaction depends on host and microbial factors, knowledge of the nature of the host immune response, host immune status and microbial factors makes it possible to predict the likely state for a given host and microbe.

The Utility of the Damage-Response Framework

The utility of the Damage-response framework is reflected in its flexibility, ability to incorporate new information and explain previous information that could not be accounted for by other views of pathogenesis and virulence. For example, the damage-response framework is able to account for why previously rare diseases, such as those caused by *Cryptococcosis neoformans* and *Pneumocystis pneumonia* occurred in epidemic proportions in individuals with HIV infection. Similarly, the damage-response framework is able to account for the emergence of *Candida albicans* as a major human pathogen in immunocompromised hosts. In addition to accounting for diseases in weak hosts, the damage-response framework can also account for diseases with excessive host responses, such as toxic shock syndrome, Kawasaki disease, allergic aspergillosis and mediastinal fibrosis. An important corollary of the damage-response framework is that infectious diseases can only occur in susceptible hosts. This concept is central to understanding whether the outcome of host-microbe interaction results in host damage, is neutral or beneficial.

Applications of the Damage-Response Framework

Education

The damage-response framework has proven to be a useful educational tool for teaching microbial pathogenesis, infectious diseases, microbiology and immunology to graduate and medical students. The advantages of teaching these disciplines based on a theoretical construct is that it leads to the use of a more universal lexicon, which enhances communication and sharpens the rigor and sophistication of research questions.

Determining the Weapon Potential of a Microbe

The lists used to categorize potential microbe-based weapons lack grounding in principles of microbial pathogenesis. The concepts of pathogenicity and virulence put forth in the damage-response framework were used to derive a standardized formula to determine the weapon potential of microbes based on the transmissibility of the microbe, the inoculum required to cause disease and the time to disease.[20,21] This formula provides a rationally based approach to assessing the potential threat that a microbe could pose as a biological weapon. In view of the corollary of the damage-response framework that infectious diseases can only occur in susceptible hosts, the damage-response framework-based formula for weapon potential provides a strategy for counteracting the threat of microbial agents of bioterror based on bolstering host immunity.

Providing Guidance on the Development of New Therapies

The Damage-response framework provides a conceptual basis for the development of new approaches to preventing and treating infectious diseases.[22,23] The functional outcome of therapies for infectious diseases is that they prevent or ameliorate the host damage that results in the state of disease. Some diseases are caused by microbe-mediated damage, while others are caused by host-mediated damage and others may result from damage due to the lack of microbially produced factors. Some diseases cannot be treated in hosts with impaired immunity and treatment of some diseases with antimicrobial agents fails to ameliorate host damage. The recognition that host damage can occur at the extremes of the host response issues a challenge to the development of therapeutics, since approaches to counteracting the damage caused by microbial factors are inherently different than approaches to counteracting damage caused by host factors. Treatment of damage due to microbial factors requires a focus on enhancing the ability of the host to eliminate the microbe or neutralize its components, whereas treatment of damage due to host factors requires a focus on reducing the inflammatory response. Each of these conditions lies on a different part of the damage-response

curve (Fig. 5). As such, intervention for a patient with damage caused by an insufficient response could require enhancement of the host response with adjuvants, cytokines or immunostimulants. In contrast, intervention for a patient with damage caused by an excessive response could require reducing the host response with steroids, immunomodulators or immunosuppressive agents. The dichotomous origins of host damage in microbial pathogenesis and infectious diseases provide the basis for a rational approach to the use of immunotherapeutic agents for infectious diseases.

Revealing New Paradigms in Host Immunity

The Damage-response framework was used to re-examine the long held view that immunity to intracellular microbes is mediated by the cellular arm of the immune system and immunity to extracellular microbes is mediated by the humoral/antibody arm.[24] A new view was put forth that antibody immunity can confer protection against a myriad of intracellular and extracellular microbes by classical and novel mechanisms that promote damage control.

Understanding the Role of the Host Microbiota in Health and Disease

Given that the damage-response framework does not view microbes as inherently pathogenic or nonpathogenic, it views the complex microbiota associated with the human host in the context of the outcome of their interaction. Hence the interaction between a healthy host and the host-associated microbiota is essential for the normal development of the immune system and for host nutrition and homeostasis. In health, the host-associated microbiota also provides a central layer of host defense by occupying a niche and preventing other microbes from establishing themselves. This community interacts with the immune system and may be regulated by immune responses to

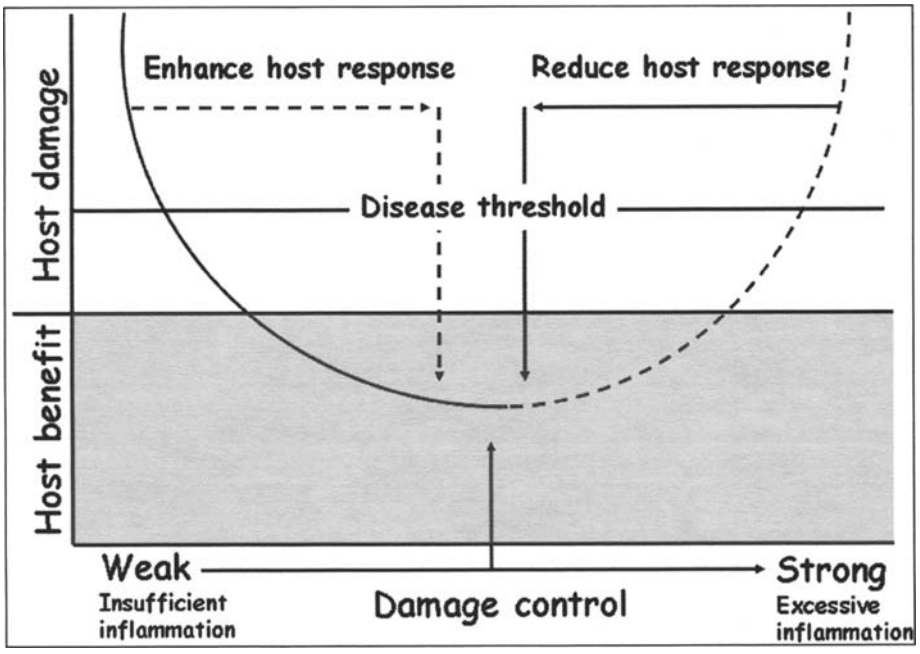

Figure 5. Use of the damage-response curve to develop approaches to therapy and prevention of infectious diseases. Host damage can occur in the setting of either weak or strong host responses. A rational approach to therapy for diseases that occur in the setting of a weak response is to enhance the host response, as shown by the dashed arrows leading to the dashed curve. A rational approach to therapy for diseases that occur in the setting of a strong response is to reduce the host response, as shown by the solid arrows leading to the solid curve.

individual microbes or complex interactions with the microbial community. Consequently, health is a condition whereby there is no disease. The state of no disease de facto includes microbes in both commensal and colonizing states, but the damage resulting from the host-microbe interactions is below the disease threshold. However, the same microbe-host interactions that are associated with health can lead to disease in situations of either a weak or excessive immune response. When an individual develops acquired immune deficiency, the same resident microbes with which they interacted in a state of normal immunity and health no longer subject to immune regulation or control and interactions with them can now cause disease. At the other end of the spectrum, an excessive immune response triggered by loss of immune regulation, or perhaps transient interaction with a microbe or allergen, could cause disease by damaging tissues in response to the presence of microbial antigens. Furthermore, immune responses to certain microbes can cause qualitative and quantitative changes in the immune response that predispose to allergic diseases. For example, experimental *C. neoformans* infection in rats does not cause clinical disease attributable to the fungus but elicits an immune response that predisposes to allergic airway disease.[25]

The simplicity and flexibility of the damage-response framework allows it to coexist easily with other views of immunity such as the 'danger'[26] and 'hygiene'[17] hypotheses. Although we note that the damage-response framework does not depend on these hypotheses for its ability to accommodate their views, its ability to incorporate them provides a measure of reassurance for its veracity. In this regard, we note that some types of host damage are analogous to the 'danger signals' postulated to elicit immune responses by Matzinger.[26] On the other hand, the damage-response framework view that health is found at the vertex of the parabola (U-curve) which corresponds to the nadir of host

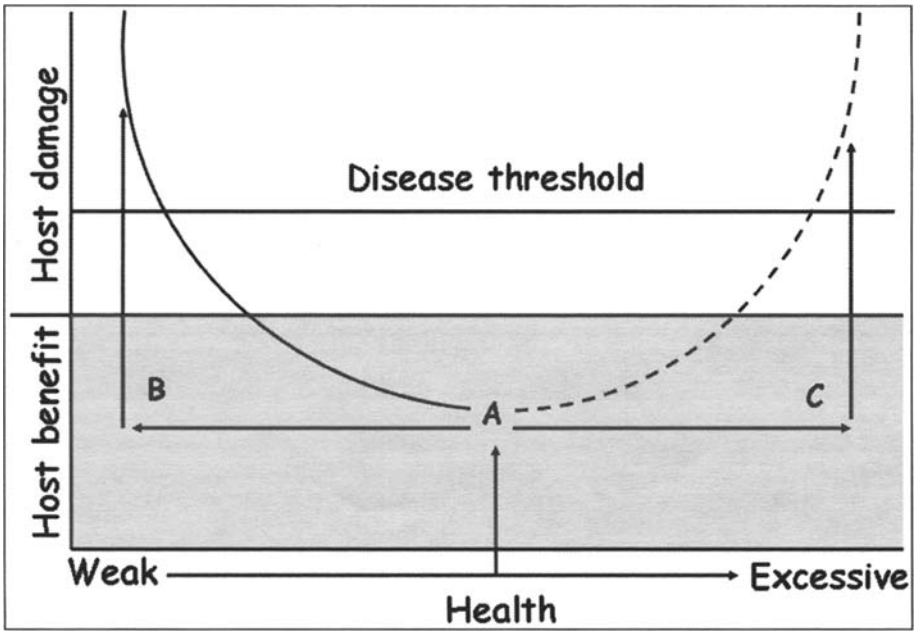

Figure 6. Use of the damage-response curve to illustrate hypothetical outcomes of human host-microbiota interactions. Host damage is portrayed as a function of the host response, whereby health is represented by an aggregate host response that controls microbiota-microbiota and microbiota-host interactions to provide a host benefit (A). Host damage occurs when the host response becomes more singular, either weak due to an insufficient response to the microbiota or the loss, disruption or dysregulation of host-microbiota or microbiota-microbiota relationships (B), or excessive due to a disproportionately strong response to the microbiota or the loss, disruption or dysregulation of host-microbiota or microbiota-microbiota relationships (C).

damage is echoed by the 'hygiene hypothesis' which posits that health requires longstanding and continued interactions with microbes to forestall the development of allergic and atopic diseases. Since the human host is in contact with thousands of microbes and for each host-microbe interaction there is an appropriate damage-response curve, one can easily imagine that the net aggregate of these responses gravitates towards a mean of minimum damage to the host. Hence, we posit that the aggregate curve of all the individual host-microbe interactions between an individual and its associated microbiota is a U-shaped curve with the condition of health requiring many types of immune responses which serve to control the microbes and to balance one another (Fig. 6).

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