Probiotics and the Immune System – Position Paper*

Modulation of host 'immunity' is one of the most commonly purported benefits of the consumption of probiotics. Reasonable, but limited, clinical evidence exists to support this concept. However, general claims regarding probiotic modulation of host immunity vastly over-state our current knowledge of both the fate of ingested probiotic products and their specific effects on molecular and cellular components of the immune system. A group* of immunologists and microbiologists assembled recently at the inaugural meeting of the International Scientific Association for Prebiotics and Probiotics to consider the status of research bearing on probiotics and the immune system. The Workshop was organized to focus on innate and acquired mechanisms postulated to be involved in functional recognition and responsiveness to probiotics encountered in the gastrointestinal tract. *The direct modulation of infectious organisms by probiotics, though a clearly important application, was generally not featured in our deliberations.* In this article, we briefly summarize the status of the field, as revealed by the presentations and discussions at the Workshop, identify the major gaps that remain in our knowledge, and outline the efforts and resources needed to fill those gaps.

Immunogenic/adjuvant properties

The immunostimulatory properties of commensal bacteria are best exemplified by studies with gnotobiotic animal models, which demonstrate that essentially all aspects of the intestinal immune system are underdeveloped in germfree animals, but rapidly restored upon the introduction of even single bacterial species (McCracken and Gaskins, 1999). Not clear, however, is the extent to which antigenic components of bacterial cell walls mediate the state of physiological inflammation that characterizes the stable association between an animal host and its resident microbiota. Similarly, there are few reports of systematic investigation of host cell responses to distinct commensal-associated molecular patterns (CAMPs) of probiotic strains, most of which are *Lactobacillus* or *Bifidobacterium* species. Studies with recombinant mutants are needed to compare, for example, intestinal epithelial cell responses to peptidoglycan or lipotechoic acid variants. In this case, it will be critical that bacterial culture conditions are standardized and that only genetically characterized probiotic strains are used. The emerging availability of genomic data will expedite efforts to identify the bioactive components displayed on the bacterial cell surface, and to derive mutants for experimental analysis. This work may lead ultimately to an understanding of the molecular basis of the variation in relative immunogenicity that clearly exists among various probiotic strains.

Fate in the GIT

It is commonly suggested that probiotics must "persist and multiply" to be effective. However, the fate of orally ingested probiotics in terms of interaction with the epithelium or other immunologically active intestinal cells has not been rigorously studied. A number of studies with a variety of probiotic strains have been conducted to determine the extent to which probiotics colonize the intestine. The combined results demonstrate conclusively that ingested strains do not become established members of the normal microbiota, but persist only during periods of dosing or for relative short periods thereafter (Fuller, 1992; Tannock, 1995; Tannock, 1997). There is also evidence that common probiotic strains differ in their degree of persistence (Fuller, 1992).

Presumably, to modulate *immunity*, probiotic organisms must reach immune cells that are endowed with recognition receptors or that are otherwise sensitive to probiotic-specific

catabolites. There is no a priori reason that introduced strains would need to persist and multiply to encounter intestinal immune cells. In fact, general acceptance that colonization is required for probiotics to be efficacious illustrates a larger deficiency in the field that being that perceived applications for probiotics are often general in nature and ill-defined. The field instead needs to consider specific immunological applications whether prophylactic or therapeutic in nature and then proceed to empirically address mechanisms by which ingested probiotic organisms might be used to prevent or treat enteric disorders. Such studies will require the formulation of detailed hypotheses regarding the fate of orally ingested probiotics in terms of interactions with specific types of host immune cells.

Given the diversity of inflammatory or immune responses that can be mounted by the intestinal epithelium, it may be that simple association of probiotics with epithelial cells, might be sufficient to trigger signaling cascades that would ultimately activate underlying immune cells in the lamina propria. Certainly much attention has been given to the adhesive properties of probiotic organisms, and an ability to adhere to host cells or mucus is commonly considered to be a requirement for probiotics. However, probiotic adhesion is another issue in need of more rigorous consideration both theoretically and empirically. The importance of adhesion for colonization is straight-forward. However, our perceptions about probiotic adhesion, especially to epithelial cells, have been derived almost entirely from in vitro studies, which are very limiting in their ability to model the intestinal epithelium. Perhaps of most importance is the fact that the surface of epithelial cells in culture is nude, while the surface of the intestinal epithelium is generally covered with a mucus coat. Our knowledge of the mucus gel and its importance as a defensive entity is significantly limited because fixation of intestinal tissues with conventional aldehyde fixatives, which are dehydrating, results in detachment and loss of surface mucus. The significance of this experimental limitation was demonstrated recently by Matsuo and coworkers (Matsuo et al., 1997). Their use of Carnoy's solution (ethanol- and acetic acid-based) enabled the preservation of surface mucus in paraffin sections of human colon samples. Two histochemically distinct mucus layers were identified, with an inner layer attached to the apical epithelial surface and continuous with intracrypt mucus. Bacteria were observed within laminated arrays of sialo- and sulphomucins in an outer layer, indicating the importance of the mucus gel in preventing direct adherence of gut bacteria to the epithelial surface. This calls into question the importance of epithelial adhesion and the physiological significance of in vitro systems that examine epithelial cell line responses to adherent probiotic organisms. Certainly much additional work is needed to determine if certain strains are able to reach and adhere to the epithelium as well as the physiological consequences of such action. These questions might also be best addressed with recombinant organisms and specific transgenic knockout mouse strains. Perhaps deserving priority would be an investigation of the extent to which epithelial adhesion might facilitate interactions with dendritic cells, which have been shown to migrate between epithelial cells and thereby 'sample' luminal contents (Rescigno et al., 2001).

Nonetheless, a large number of in vitro studies have been reported, which examined in one manner or another epithelial cell responses to adherent probiotic strains (Borchers et al., 2002). These studies demonstrate the ability of probiotic strains to upregulate a variety of cytokines and inflammatory molecules, however, the physiological context of the observed responses is rarely considered. Once again, it would appear most useful to design the in vitro studies with a particular intestinal disorder or physiological process in mind such that specific immunologic or inflammatory functions or molecules could be targeted.

Translocation
A key consideration is the effect of bacterial adhesion on translocation across the epithelium. Routine translocation of commensal bacteria to mesenteric lymph nodes has been clearly demonstrated (Berg & Owens, 1979, Berg 1995, 1999; Gautreaux et al., 1994), and presumably is central to the developmental activation of the intestinal immune system. Furthermore, Berg and colleagues have demonstrated among a variety of intestinal bacteria, an inverse relationship between degree of adhesiveness and degree of translocation (RD Berg, personal communication). This work should be expanded to screen a wide range of well characterized probiotic strains, and to also take into consideration the possibility that some strains may be capable of modulating tight junctions and thereby cross the epithelium. Also needed are non-invasive methods for measuring bacterial translocation before the importance of this process is understand in terms of immunological responsiveness to probiotic or other commensal bacteria In the meantime, it appears that 'physiological translocation' (i.e., to MLNs) would be a more desirable trait of candidate probiotic strains than adhesion to the epithelial surface.

On the other hand, bacterial adhesion to M cells covering Peyer's patches would be expected to enable the activation of IgA responses, which depending on context might be a desired outcome. This area of research is also in critical need of further investigation. Unfortunately, M cells cannot be propagated in primary culture and physiologically relevant M cell lines do not exist. Recombinant strains genetically designed to target M cells and which are labeled by one means or another for in situ identification will be required to initiate investigation of M cell and host IgA responses to probiotic organisms. These studies should also employ transgenic mouse strains in which specific immune components have been genetically ablated to systematically define the cellular and molecular basis of host responsiveness to probiotics.

Genetically-tagged bacterial strains will also be crucial for determining the regions of the gastrointestinal tract that are most immunologically responsive to ingested probiotic strains, another key consideration that is fully undefined at present. Given the central role of Peyer's patches for the development of secretory IgA, it follows that probiotic strains targeting M cells should be identified for applications that seek to bolster intestinal immunity. On the other hand, probiotic strains with an affinity for the colonic epithelium and also possessing anti-inflammatory properties would be needed for the treatment of large intestinal inflammatory disorders such as ulcerative colitis. At this stage, however, these are only theoretical considerations, as the availability of standardized reagents and experimental conditions are generally not in place for empirical research.

Oral tolerance and allergic diseases
Probiotics are often targeted for neonatal applications, which brings up critical questions pertaining to the timing of and mechanisms by which intestinal bacteria contribute to the establishment of tolerance versus sensitization to ubiquitous antigens. Neonatal immune responses to environmental antigens are generally skewed toward a Th2-type cytokine profile, which typifies allergic diseases (Prescott et al., 1998; 1999). Allergic diseases have increased substantially in developed countries during recent decades, which has led to the formulation of the "hygiene hypothesis". This hypothesis ascribes the increase in allergic disease to increased emphasis on hygiene, which reduces the exposure of neonates to microbial stimuli thereby skewing immune responses toward a Th2 versus a Th1 cytokine profile (Erb, 1999; Matricardi & Bonini, 2000). Intestinal colonization with commensal bacteria is critical for the establishment of oral tolerance (Weiner 1997), which has heightened interest in the potential use of probiotics

to prevent allergic diseases in neonates. Justifying this focus is a recent clinical study, which demonstrated a highly significant reduction in the frequency of atopic eczema in infants who either nursed mothers that were taking a *Lactobacillus* GG supplement or who received themselves *Lactobacillus* GG (Kalliomäki et al., 2001). The immunological basis of this outcome was not determined, but the promise of these results necessitate focused efforts to define the cellular and molecular responses of the intestinal immune system to commensal bacteria during early colonization, as well as changes induced by probiotic supplementation. The wide range of existing animal models, particularly transgenic knockout mice with specific cellular or molecular deficiencies (e.g. B- and T-cell deficient animals), have not been used adequately to investigate immunological responses to either commensal or probiotic bacteria. A number of key questions or goals could be addressed currently with these models including: 1) identification of the developmental windows, which are most sensitive to immunological manipulation; 2) the selection of well defined immunogenic versus tolerogenic probiotic strains; and 3) the identification of standard immunological biomarkers that could be measured in human clinical studies such as the atopic eczema study described above.

Immune cells: role of T and accessory cells
Tolerance and homeostasis in the intestine is maintained by specialized subsets of lymphocytes. Subsets of CD4+ T cells have drawn most of the attention so far and several phenotypes have been described, depending on the type of cytokines or surface molecules they express. At least three subsets of regulatory CD4+ T cells have been characterized that may play a role in gut homeostasis (Th3, Tr1, CD25+) (Toms and Powrie, 2001). There also seems to be a role for other T cells types, including NK T cells (Saubermann et al., 2000) and gamma delta IEL (Fujihashi et al., 1996; Ke et al., 1997), but more studies are needed to clarify their contribution. A very recent study has described the presence in the gut of evolutionarily conserved mucosal-associated invariant T cells that are MR1-restricted and require the presence of the commensal flora for their expansion in the lamina propria (Treiner et al., 2003), but their function remains to be elucidated.

The microbiota has a positive impact on immune regulatory functions of the gut, but disruption of these immune regulatory functions by an imbalanced microbiota may lead to exacerbated effector responses and chronic inflammatory diseases. Some interest has therefore recently emerged to address the potential role of probiotics in the induction (or restoration) of regulatory-type immune responses in the gut.

Very few studies have actually addressed the modulation of lymphocyte subsets by probiotics. Experiments in rats and mice using several strains of lactobacilli have shown an increase in proportions of CD25+ cells in the lamina propria (Herias et al., 1999) and a decrease in T cell reactivity (Kirjavainen et al., 1999b; Kirjavainen et al., 1999a; Mike et al., 1999). A few *in vitro* studies have suggested that unknown components of lactobacilli may have anti-proliferative effects on T cells and suppressive effects on cytokine production by T cells (Pessi et al., 1999; Pessi et al., 2001; von der Weid et al., 2001). Interestingly, one study showed a concomitant emergence of a Tr1-like cell population *in vitro* (von der Weid et al., 2001).

It is clear that the nature of the T cell response that takes place in the gut is regulated by the local DC populations that interact with these cells (review by Kelsall et al., 2002). Several studies demonstrated that mucosal DC differ from systemic DC in their capacity to either suppress or prime immune responses, respectively (Everson et al., 1998; Iwasaki and Kelsall, 1999a; Iwasaki and Kelsall, 1999b). A recent paper has shown that microbial compounds can

selectively induce Th1 or Th2 polarizing signals in DC depending on their structure and origin (De Jong et al., 2002). Probiotics clearly have a role to play in modulating the maturation and function of dendritic cells, as recently demonstrated *in vitro* with several lactobacillus strains (Christensen et al., 2002).

All these studies were made using "reductionist" *in vitro* systems that were all different from each other and where many potential players of the mucosal regulatory response were lacking. Therefore many questions remain. For example, what is the contribution of the other numerous T cells types present in the lamina propria and in the epithelium, such as NK T cells, gamma delta IEL, and the newly described MR1-restricted mucosally associated invariant T cells? Most importantly, the key role of the epithelial cell in the whole process should be better integrated using appropriate co-culture *in vitro* systems (Schiffrin and Blum, 2002). What components of the bacteria interact with the DC and what is the relative importance of known PRR receptors? In parallel, *ex vivo* studies that target the individual cellular components of the mucosal immune system are now feasible, using laser micro-dissection techniques coupled to genomics (Hooper et al., 2001; Hooper and Gordon, 2001). This approach should allow us to have a more global approach and link patterns of signals induced by probiotics with those associated with tolerogenic or pathogenic mucosal responses.

Secretory IgA

Secretory IgA (SIgA) is the most abundantly produced immunoglobulin at the surface of mucous membranes in mammals. SIgA contributes to specific immunity against invading pathogenic microorganisms. In the gut, SIgA production depends on intricate mechanisms involving antigen sampling by M cells (Neutra, 1999), processing by underlying antigen-presenting cells (APC; Kelsall and Strober, 1997), T cell activation (Lycke, 1998) and B cell switch in the Peyer's patch and neighboring lamina propria (Brandtzaeg et al., 1999). Multiple cytokines including interleukin(IL)-4, TGF-beta, IL-5, IL-6, IL-10 are instrumental to intestinal SIgA production, yet discrepancies between *in vitro* and *in vivo* data remain a matter of controversy as to their physiological functions. As the same set of cytokines are required for maintaining tolerance and IgA switch and production, this established a link that can partly explain why mucosal SIgA are considered as non-inflammatory in the mucosal environment (Russell et al., 1997).

In what can appear as a paradox, intestinal SIgA is also spontaneously induced by the presence of non-pathogenic commensal microorganisms. Changes in the intestinal flora result in induction of specific SIgA responses through a pathway independent of T cell help and maturation (Macpherson et al., 2000). In contrast to bacterial mucosal antigen, this makes sense since control of the endogenous flora requires a broad spectrum of reduced affinity SIgA. The adaptive SIgA responses to the intestine flora could limit penetration from the intestine into the body, and contribute to homeostasis (Shroff et al., 1995). Indeed, the gut flora was shown to induce and maintain oral tolerance in experimental animal models (Gaboriau-Routhiau and Moreau, 1997). In addition, commensals act as an important non-specific antigenic stimulus for the maturation of GALT (Cebra et al., 1998).

Probiotics have been shown to boost IgA antibody responses and therefore trigger intestinal immune exclusion and subsequent elimination (Fukushima et al., 1998; Tejada-Simon et al., 1999; Fang et al., 2000; Cukrowska et al., 2002). Moreover, probiotic bacteria contribute to modulate the host's immune responses to potentially harmless antigens by prompting down-regulation of hypersensitivity reactions (Sutas et al., 1996a; Sutas et al., 1996b; Pessi et al., 2000; Pessi et al., 2001). The mechanisms whereby probiotics modulate immune responses leading to

SIgA activation or tolerance appear to be highly dependent on the strains. However, to date, the use of multiple assay models to monitor their performance prevents however to establish consensus rules as to the cellular and immune mediators involved. As discussed above, antigen phagocytosis and processing by APC in the Peyer's patch (dendritic cells and macrophages) promotes mucosal induction of IgA. What is thus the possible contribution of probiotics in acting on such multicellular events? Whether probiotics can interfere with an inflammatory signaling pathway in the intestinal mucosa as this has been reported for non pathogenic salmonellae (Neish et al., 2000) remains to be explored.

An intriguing recent study provides evidence that a 30-mer peptide comprising amino acids 38-67 from human secretory component in SIgA exhibits prebiotic properties when incubated with various bifidobacteria strains (Liepke et al., 2002). The bidfidobacterial growth was stimulated 100 times more effectively than with equimolar amounts of the carbohydrate N-acetylglucosamine. This suggests that induction of SIgA by probiotics might in turn lead to the stimulation of bacterial growth in what can be seen as some sort of a loop mechanism. This also indicates that the bifidogenic effect of milk containing SIgA is not solely due to its sugar content as generally thought. This argues in favor of a relevant function for free secretory component found in mucosal and gland secretions (Cleveland et al., 1991; Crottet et al., 1999).

Recombinant lactic acid bacteria with enhanced health effects
The potential of lactic acid bacteria to act as live mucosal delivery system has been investigated during the last decade (For recent reviews see Corthier and Renault, 1999; Thole et al., 2000; Seegers, 2002; Wells and Mercenier, *In press*). Even though strain-specific immunoadjuvant properties have been demonstrated for a number of *Lactobacillus* species (see above), the intrinsic antigenicity of lactic acid bacteria seems to be rather low by mucosal routes. This has not prevented to use these microorganisms as effective carriers for protective antigens. The most complete studies have been carried out with the C subunit of the tetanus toxin (TTFC). Both persisting – i.e. *Streptococcus gordonii, Lactobacillus plantarum* and *Lactobacillus casei* - and non-persisting – i.e. *Lactococcus lactis* - species have been investigated. The strains producing sufficient levels of the antigen were shown to induce high levels of serum IgG after nasal or intragastric administration. In the best cases, these immune responses were shown to be protective. Also, local TTFC-specific IgA were induced (for a recent review see Mercenier et al., 2000). In the course of this work, Steidler et al. (1998) demonstrated that the immune responses could be enhanced by the co-delivery of IL2 or IL6 and TTFC. This approach was further extended by the construction of recombinant *L. lactis* strain secreting murine IL10. The authors successfully demonstrated that these strains were able to prevent or treat inflammation in murine colitis models (Steidler et al., 2000). Notably, this effect was obtained with much lower doses of IL10 than those required when the interleukin was used as a single therapeutic agent. Steidler et al. (unpublished results) further constructed a safe (no antibioresitance marker and chromosomally integrated transgene) biologically contained strain secreting the human IL10. Authorization to conduct a phase 1 clinical trial with this strain (targeting IBD) has been obtained recently in the Netherlands. In the meantime, production and mucosal delivery of different types of health promoting molecules has been achieved in lactic acid bacteria such as ScFv antibodies, allergens or digestive enzymes. Targeted diseases included microbial infections such as vaginal candidiosis (Beninati et al., 2000) and dental caries (Krüger et al., 2002), allergies (Kruisselbrink et al., 2001; Chatel et al., 2001), autoimmune diseases (Maassen et al., 1999), and metabolic defects such as pancreatic insufficiency (Drouault et al., 2002). Much

efforts are presently being devoted to improve the efficiency of lactococci or lactobacilli as delivery system. Therefore, mutants are generated which release intracellular compounds more efficient (Walker and Klaenhammer, 2001) or which present altered delivery properties (Grangette, Hols, Delcour and Mercenier, unpublished). It might be foreseen that immunization studies might shed a new light on the probiotic field, as the biomarkers in these studies are well defined and relatively easy to follow.

Although recombinant strains would not be accepted today in functional foods, their future use in therapeutic approaches can be foreseen provided that the benefit /risk balance is positive. It might be expected that such strains would be formulated as capsules or powder preparations and will be prescribed by medical doctors. By no means are they intended to be included in retail products. This novel approach is rather promising as grafting the gene encoding for a therapeutic molecule could enhance the natural health beneficial properties of a strain. For example, strains naturally exhibiting anti-*Helicobacter pylori* properties could be designed to produce a protective *H. pylori* antigen. In addition to these targeted constructions, mutants in specific genes encoding for potential probiotic functions (such as adhesion to mucus, resistance to acid, specific cell wall components etc) could be engineered and compared to their wild type counterpart in a series of *in vitro* and *in vivo* models. This strategy should help in unraveling the mechanisms underlying the cross talk between probiotic bacteria and their host. Notably, the available and upcoming genome information will largely facilitate this approach. Moreover, fluorescently labeled bacteria (Geoffroy et al., 2000) could be used to better understand the fate of the bacteria after ingestion and may help to analyze which are the major immune cells that recognize and process them.

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