Old Herborn University Seminar Monograph 23:
Intestinal microbiomics: novel indicators of health and disease.
Editors: Peter J. Heidt, Johannes Snel, Tore Midtvedt, and Volker Rusch.
Old Herborn University Foundation, Herborn-Dill, Germany: 65-74 (2010).

HOST-MICROBE INTERACTIONS: TOWARD THE IDENTIFICATION OF MECHANISMS

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SUMMARY

The microbial community resident in the gastrointestinal tract influences the host organism in many ways. It has been recognized that the intestinal microbiota supports important physiological functions in the host and thereby contributes to disease prevention. However, the gut microbiota also plays a role in the development of certain diseases, such as inflammatory bowel disease and colorectal cancer. Many effects of the intestinal microbiota can be attributed to the immense catalytic potential of this microbial community. Beneficial activities include the activation of potentially chemopreventive substances ingested with the diet, while adverse effects are due to the formation of genotoxic or carcinogenic compounds. On the one hand intestinal bacteria contribute to the activation of lignans and isoflavones, which have been implicated in the prevention of breast and prostate cancer. On the other hand the deglycosylation of dietary arbutin by intestinal bacteria leads to the formation of mutagenic hydroquinone. The application of metagenomics revealed previously unknown correlations between the host and its gut microbiota, such as a role of the gut microbiota in obesity. The molecular mechanisms underlying such microbe-host interactions are largely obscure. However, a combination of novel tools such as the 'omics' technologies and bioinformatics, as well as classical microbiological methods and gnotobiology will help us to unravel piece by piece the molecular basis of these interactions.

INTRODUCTION

It has increasingly been recognized that the gut microbiota has a major impact on host physiology and intestinal function. However, the molecular mechanisms underlying gut microbiota-mediated effects on the host are far from being understood. Even less knowledge exists on how endogenous and exogenous factors shape the composition and function of the gut microbiota and how this in turn affects host physiology. This is largely due to the complexity of the gut microbiota and its high individual variability. Recent advances in high throughput sequencing, transcriptome and microbiome analysis as well as bioinformatics enabled the recognition of previously unknown functions of the gut microbiota and provided new insights into how intestinal bacteria exert their effect on the host. However, in spite of all of these great advances it

also has become clear that we are still far away from understanding the molecular basis of the multi-faceted host-microbe interactions. We therefore need to keep on exploring this fascinating ecosystem with a wide range of experimental approaches.

REVIEW AND DISCUSSION

Roles of intestinal bacteria in disease

Intestinal bacteria have been implicated in a number of diseases, including inflammatory bowel disease, colon cancer and, more recently, the metabolic syndrome. Inflammation of the bowel as observed in Crohn's disease and ulcerative colitis, becomes manifest in the susceptible host whose immune system has not acquired tolerance toward the antigens of commensal gut bacteria (*Sartor*, 2006). Whether, and to which extent, specific organisms play a role in the manifestation of this disease is still unclear. In any case, inflammatory bowel disease exemplifies the intricate relationship between the host and its microbiota, in particular its interaction with the host immune system.

Gut microbiota and inflammation

We studied the intestinal microbiota composition in the IL-10-deficient mouse (IL-$10^{-/-}$), a widely accepted model for chronic colitis. Molecular microbial population analysis revealed a decreased microbial diversity in the inflamed gut, with *Escherichia coli* and *Blautia producta* becoming the dominant bacterial species (*Wohlgemuth* et al., 2009). Phylogenetic grouping revealed that all mice (IL-$10^{-/-}$ and wild-type) were colonized by one single *E. coli* strain with the serotype O7:H7:K1. We detected a high number of virulence- and fitness-associated genes in this strain's genome, which possibly are involved in the organism's adaptation to the murine intestine. When this strain was introduced into germ-free mice together with two other *E. coli* strains, the isolate overgrew its competitors. However, we found no evidence that the strain causes gut inflammation in conventional animals. We therefore conclude that the observed growth stimulation of *E. coli* in gut inflammation is rather a consequence than cause of the disease.

Formation of genotoxic compounds

The role of the intestinal microbiota in colon cancer development is more related to its immense catalytic potential which has been compared to that of the liver. Intestinal bacteria convert both endogenous and exogenous substrates. The bacterial conversion in the intestine of some of these substrates may lead to the formation of genotoxic compounds. Secondary amines and phenols are typical products of the bacterial breakdown of proteins in the distal colon (*Macfarlane* et al., 1988). In conjunction with nitrite they may lead to the formation of nitrosamines and *p*-nitrosophenol (*Kikugawa and Kato*, 1988), both of which are highly carcinogenic.

Genotoxic compounds may also arise from the bacterial conversion of non-nutritive dietary components, such as arbutin that is found in pears, coffee and wheat. We investigated the deglycosylation of arbutin by intestinal bacteria, which convert this compound to hydroquinone, a mutagenic and therefore potentially carcinogenic substance

(*Blaut* et al., 2006). Furthermore the conversion of neoglucobrassicin, a glucosinolate found in vegetables such as broccoli and pak choi, leads to the formation of DNA adducts in the mucosa of the upper part of the intestinal tract when consumed raw, but preferentially in the colonic mucosa when consumed after cooking. In the former case plant-derived myrosinase catalyzes the cleavage of the compound while in the latter case preferentially intestinal bacteria are assumed to catalyze this activation because no DNA adducts are found in germ-free animals. These examples show that the intestinal microbiota catalyzes a wide range of reactions that may have adverse health effects on the host.

Roles of intestinal bacteria in health

Contribution to intestinal barrier

Intestinal bacteria contribute to the intestinal epithelial barrier, which prevents the translocation of undesirable components from the gut lumen into the underlying host tissues and the circulation (*Berg*, 1996). This barrier effect is of utmost importance as it largely prevents the invasion of intestinal tissue by pathogens and ensuing disease. Competitive exclusion of invaders impedes their establishment and persistence in the ecosystem unless they are capable of successfully competing for nutrients and binding sites. The intestinal microbiota also fortifies the intestinal barrier by keeping the mucosal immune defence in a state of alertness. Various host cells possess so-called pattern recognition receptors (*Cario*, 2005) that sense and identify bacterial cells based on their characteristic cell components, and, following the detection of pathogens they trigger an immune response.

Formation of short-chain fatty acids

Another important function of the gut microbiota lies in its ability to process non-digestible dietary as well as endogenous components. Dietary fibre, which includes a wide range of oligomeric and polymeric carbohydrates, escapes digestion in the small intestine and, upon reaching the colon, undergoes fermentation by intestinal bacteria. Dietary fibre not only is the main source of energy for intestinal bacteria but also leads to the main products of bacterial fermentation, preferentially the short chain fatty acids acetate, propionate and butyrate (*Mortensen* and *Clausen*, 1996). Besides providing energy to the host it has been recognized that short chain fatty acids are important for the maintenance of a healthy colonic mucosa. In particular butyrate has been implicated in the prevention of ulcerative colitis and colon cancer owing to its effect on epithelial cell proliferation and cell differentiation (*Andoh* et al., 2003).

Bio-activation of dietary components

Intestinal bacteria also play a major role in the activation of non-nutritive dietary components such as polyphenols. These stem from dietary plants and include tannins, lignans and flavonoids. The latter two groups are in the focus of intense research because both epidemiological and experimental data indicate that they may play an important role in disease prevention. For example, isoflavones and lignans have been implicated in the prevention of breast and prostate cancer, osteoporosis, menopausal symptoms, and cardiovascular diseases. Following their oral intake, polyphenols may undergo trans-

formation by intestinal bacteria which in turn influences both their bioavailability and bioactivity in the human intestinal tract.

The conversion by intestinal bacteria of non-nutritive dietary components, such as polyphenols, does not appear to be essential for the gut microbial community as a whole. This may be deduced from the fact that the conversion of some of these substances is not observed in all individuals but only in a subpopulation. For example, only 30-50% of human subjects excrete equol in their urine in response to the consumption of soy, which is a major source of daidzein (*Atkinson* et al., 2005). Intestinal bacteria convert the isoflavone daidzein and the lignan secoisolariciresinol-diglucoside (SDG) to equol and enterolactone, respectively. The latter have been implicated in the prevention of sex hormone-related cancers, osteoporosis and the alleviation of menopausal symptoms (*Thompson* et al., 2005). Isoflavones and their metabolites may bind to oestrogen receptors and exert agonistic or antagonistic effects (*Scalbert* et al., 2005).

Microbial conversion of lignans

Lignans undergo activation by intestinal bacteria to adopt estrogenic and anti-oxidant activities. We recently isolated and identified human intestinal bacteria involved in lignan activation and characterized all steps leading from the plant lignan SDG to enterodiol (ED) and enterolactone (EL) (*Clavel* et al., 2005, 2006a,b). We described two of these isolates as new species: *Clostridium saccharogumia* and *Lactonifactor longoviformis*, with the latter representing a new genus (*Clavel* et al., 2007). The ability of faecal bacteria to convert SDG to the oestrogen-like metabolites ED and EL was found to be widely distributed among humans. Women tended to show higher concentrations of both ED- and EL-producing organisms.

Microbial conversion of isoflavones

To study the conversion of isoflavones in more detail, we isolated and characterized two bacterial strains from the mouse intestine and from human faeces, respectively (*Matthies* et al., 2008, 2009). The strains converted daidzein via dihydrodaidzein to equol. Likewise, the new isolates formed 5-hydroxy-equol from genistein via dihydrogenistein. The conversion of daidzein and genistein depended on the pre-incubation with these isoflavones, indicating that the corresponding enzymes are inducible. Both isolates are new species belonging to the *Coriobacteriaceae*.

These few examples highlight the profound impact of the intestinal microbiota on the host metabolism and how nutrition affects this correlation. However, although we are aware that the intestinal microbiota affects the host in many ways it is very likely that only a small proportion of the effects conferred by gut bacteria on the host have been recognized. We know even less on how these effects are brought about. There are numerous questions that have not yet been answered. These include: Which nutritional, environmental and host factors govern the development of the microbial community in the newborn? What explains the immense microbial diversity of the gut microbiota at the species and strain level in spite of similar key functions? How do host and nutrition factors affect the composition and functional activity of the gut microbiota?

The metagenomic approach

The advent of the 'omics' technologies and the ease and speed of high-throughput sequencing has opened new opportunities for investigating the gut microbiota and its interactions with the host. Sequencing of all genes present in the genomes of all members of the gut microbial community in different individuals is part of the Human Microbiome Project (*Turnbaugh* et al., 2007). This project preferentially aims to determine whether individuals share a core human microbiome and whether changes in this microbiome can be correlated with health and disease. Metagenomics is thought to provide relatively unbiased information about the community structure and its functional potential (*Hugenholtz* and *Tyson*, 2008). The application of a metagenomic approach to the human gut microbiota revealed previously unknown correlations. For example, the observation that the association of germ-free mice with a microbiota obtained from obese mice results in a greater increase in total body fat than with a microbiota from lean mice prompted an investigation using a metagenomic approach. As a result, the gut microbiota was identified as a factor that may contribute to the pathophysiology of obesity. Taxonomic analyses in the obese host revealed a higher proportion of intestinal bacterial cells belonging to the Firmicutes and Actinobacteria than to the Bacteriodetes. This has been demonstrated for both mice and humans (*Turnbaugh* et al., 2006, 2009). These taxonomic differences in the microbiome of the obese host were accompanied by an enrichment of genes encoding enzymes involved in the breakdown of dietary polysaccharides that escape digestion in the small intestine and in enzymes involved in lipid and amino acid metabolism. These genes were proposed to comprise a set of microbial biomarkers characteristic of the gut microbiome of the obese host.

Although only few laboratories have the equipment and the powerful bioinformatics at their disposal to analyze the huge amount of data being produced in the course of such studies, it has to be acknowledged that the metagenomic approach offers the unique opportunity to discover new correlations between host and gut microbiota. However, usually the metagenomic approach does not reveal the molecular mechanisms underlying an observed correlation between a given physiological or pathophysiological status of the host and an enrichment of certain genes, which reflect the microbiota composition and the metabolic and functional potential of the microbial community. Another drawback of metagenomics lies in the fact that the gap between characterized and hypothetical proteins is getting bigger as more and more sequence data become available (*Hugenholtz* and *Tyson*, 2008).

The study of host-microbe interactions

'Omics' technologies in conjunction with the use of animal models with a defined microbial status have lent momentum to the study of host-microbiota interactions and their underlying molecular mechanisms. Various publications demonstrate the power of this approach. For example, it has been shown that the gut microbial community shapes the intestinal environment: In mice, intestinal bacteria are essential for the continuation of a differentiation

program which is initiated after birth and leads to the fucosylation of the small intestinal epithelium; one intestinal species, namely *Bacteroides thetaiotaomicron*, was sufficient to induce the expression of fucosyltransferase in small epithelial cells (*Bry* et al., 1996). Other recent investigations indicate that the gut microbial community affects both nutrient harvest and energy metabolism of the host (*Backhed* et al., 2004, 2005, 2007). The influence of the gut microbiota on the host energy metabolism is considered to be of particular relevance in view of the epidemic increase in obesity.

The availability of the complete genomic sequence of man and mouse prompted investigations into host gene expression in different intestinal tissues in response to bacterial colonization of the gastrointestinal tract. Germ-free mice were mono-associated with *Bacteroides thetaiotaomicron* (*Hooper* et al., 2001), a numerically dominant member of the intestinal microbiota, which is known for its versatility in the use of complex carbohydrates as energy substrates. A large variety of genes involved in nutrient absorption, detoxification of xenobiotics, intestinal maturation, mucosal barrier function and innate immunity were shown to undergo changes in their expression in response to the association with this organism. A wide range of genes involved in intestinal maturation, mucosal barrier function, nutrient uptake and conversion of xenobiotics, were increased by more than two-fold in response to the association with *B. thetaiotaomicron*. The increased expression of the genes encoding Na^+/glucose co-transporter (SGLT1), pancreatic lipase-related protein (PLRP-2), co-lipase, liver fatty acid binding protein (L-FABP) and apolipoprotein A-IV indicate that *B. thetaiotaomicron* improves nutrient absorption. This is in line with the observation that conventional rodents gain 40% more body fat than their germ-free counterparts in spite of a lower consumption of a standard rodent chow diet (*Backhed* et al., 2004).

The genomes of a number of bacterial species relevant for the human intestinal tract have been sequenced. Analysis of the *B. thetaiotaomicron* genome indicated that this organism has an arsenal of enzymes devoted to the utilization of a large variety of carbohydrates (*Xu* et al., 2003). A comparison of gene-expression profiles of *B. thetaiotaomicron* from mice mono-associated with this organism and fed either a fibre-free diet or a diet containing fermentable fibre, indicated that the bacteria from the mice fed the former diet primarily expressed genes involved in the breakdown of host-derived substrates such as mucins and chondroitin sulphate (*Sonnenburg* et al., 2005). In contrast, the bacteria obtained from the mice fed the latter diet expressed genes involved in the degradation of fermentable dietary fibre such as resistant starch and pectin. These data indicate that host and dietary factors influence bacterial gene expression in such a way that intestinal bacteria are capable of optimally adapting to a given metabolic situation.

The role of intestinal bacteria in fortifying the mucosal barrier and thereby improving colonization resistance has many facets, one of which has been investigated in more detail in mice mono-associated with *B. thetaoitaomicron*. Colonization of germ-free mice with this organism, but also lipopolysaccharides from *Salmonella* induce the expression of Angiogenin-4 (Ang4), which originally was assumed to play a role in angiogenesis but later on Ang4 was shown to be a cryptdin, an antimicrobial defensin produced by Paneth cells and secreted into the gut

lumen (*Hooper* et al., 2003). Ang4 is effective against a number of Gram-negative and Gram-positive bacteria such as *Listeria monocytogenes* and *Enterococcus faecalis*. This mechanism contributes to the maintenance of epithelial integrity and helps to protect the host from detrimental environmental effects. Once the barrier becomes disrupted, bacterial and food antigens have access to the sub-mucosa. Here they may induce an inflammatory response which, if uncontrolled, may lead to inflammatory bowel diseases.

These examples show that for mechanistic studies it is necessary to dissect the complex interactions between the host and its microbiota. This can be accomplished by applying a reductionistic approach, which helps to minimize the number of confounding parameters. In addition it will remain necessary to investigate new bacterial activities which may be considered minor but which may have important consequences for the host.

Bacterial response to the host environment

The effects of the gut microbiota on the host have been studied extensively. In contrast, the microbial response to host factors has hardly been considered although this response may have implications for the host. We took advantage of a reductionistic animal model for investigating how an intestinal bacterium adjusts its physiology to the specific conditions in the gastrointestinal tract. We associated germ-free mice with commensal *Escherichia coli* as a simplified model of host-bacteria interactions. We analyzed the bacterial adaptation to the gut environment by a proteomic approach using two-dimensional gel electrophoresis followed by electron-spray ionization-tandem mass spectrometry (*Alpert* et al., 2005). We characterized 60 arbitrarily chosen protein spots and identified 50 unique bacterial proteins. Their ascribed functions suggest that the host-associated bacteria adapt their metabolism to the simultaneous use of a wide spectrum of substrates available in the intestinal tract. This differs completely from the situation in nutrient-rich media where the metabolism of *E. coli* is strictly regulated so that preferably only one substrate is utilized at a time. We detected ten proteins with unknown or poorly characterized physiological functions and three proteins whose existence so far had been inferred from predictions only. We assume that some of these proteins play a role in the bacterial adaptation to the host environment (*Alpert* et al., 2009). We are now in the process of producing *E. coli* strains in which the genes of interest have been knocked out in order to study the effect of these gene knockouts on the cells' ability to colonize germ-free mice and to compete with the corresponding wild type strain.

CONCLUSIONS

Interest in the intestinal microbiota has been largely triggered by the awareness that this microbial community affects the health status of the host organism. Technological progress enabled the development of novel experimental approaches, such as the metagenomic approach, which revealed correlations between host health and the microbiota. Observations include the enrichment or

the depletion of members of the bacterial community or sets of genes. However, which mechanisms underlie the observed effects has largely remained obscure. Similarly, there is little knowledge on how host and nutrition factors shape the intestinal microbiota. To get mechanistic insights into these processes it is mandatory to improve the knowledge base by combining a whole range of methods and tools. Not only will it be necessary to keep on isolating new intestinal bacteria with relevant activities, but we also need to diminish the proportion of genes with unknown functions. All of this involves classical microbiology and biochemistry. This in conjunction with the use of gnotobiotic and knockout animals will bring us closer to a better understanding of the mechanisms underlying host-microbe interactions.

ACKNOWLEDGMENT

I would like to gratefully acknowledge the excellent contributions of my co-workers and the financial support by the DFG and by the BMBF.

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