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REVIEW

The role of gut microbiota (commensal bacteria) and the mucosal barrier in the pathogenesis of inflammatory and autoimmune diseases and cancer: contribution of germ-free and gnotobiotic animal models of human diseases

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Metagenomic approaches are currently being used to decipher the genome of the microbiota (microbiome), and, in parallel, functional studies are being performed to analyze the effects of the microbiota on the host. Gnotobiological methods are an indispensable tool for studying the consequences of bacterial colonization. Animals used as models of human diseases can be maintained in sterile conditions (isolators used for germ-free rearing) and specifically colonized with defined microbes (including non-cultivable commensal bacteria). The effects of the germ-free state or the effects of colonization on disease initiation and maintenance can be observed in these models. Using this approach we demonstrated direct involvement of components of the microbiota in chronic intestinal inflammation and development of colonic neoplasia (i.e., using models of human inflammatory bowel disease and colorectal carcinoma). In contrast, a protective effect of microbiota colonization was demonstrated for the development of autoimmune diabetes in non-obese diabetic (NOD) mice. Interestingly, the development of atherosclerosis in germ-free apolipoprotein E (ApoE)-deficient mice fed by a standard low-cholesterol diet is accelerated compared with conventionally reared animals. Mucosal induction of tolerance to allergen Bet v1 was not influenced by the presence or absence of microbiota. Identification of components of the microbiota and elucidation of the molecular mechanisms of their action in inducing pathological changes or exerting beneficial, disease-protective activities could aid in our ability to influence the composition of the microbiota and to find bacterial strains and components (e.g., probiotics and prebiotics) whose administration may aid in disease prevention and treatment.
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INTRODUCTION

The majority of epithelial surfaces of our body, such as the skin and mucosa, are colonized by a vast number of microorganisms; these represent the so-called normal microflora, the microbiota. The microbiota comprises mainly bacteria; however, viruses, fungi and protozoans are also present. Our microbiota contains trillions of bacterial cells, 10 times more cells than the number of cells constituting the human body. Most of the commensal bacteria are symbiotic; however, after translocation through the mucosa or under specific conditions, such as immunodeficiency, commensal bacteria could cause pathology. Bacteria are present at anatomical locations that provide suitable conditions for their growth and proliferation. Skin is predominantly colonized by bacteria in the skin folds. The upper airways, particularly the nasopharynx, harbor bacteria, as do some mucosal surfaces of the genital tract, although the greatest number of bacterial cells is found in the digestive tract. The oral cavity (tongue, teeth and periodontal tissues) harbors high numbers of bacteria (10^{12}). The stomach has only 10^3–10^4 bacteria, the jejunum harbors 10^5–10^6 bacteria and the terminal ileum harbors 10^8–10^9. However, the largest number of bacterial cells is found in the large intestine (10^{11} per gram

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of intestinal content). A considerable portion, about 70%, of this microbial cosmos inside our body is composed of bacteria that cannot be cultivated by current microbiological methods. Microbial groups have been found to develop in close parallel with the human body and to depend on the physiological environment in unity with their hosts; hence, like most other higher organisms, humans are, in fact, supraorganisms. Our microbiota represents a complex ecosystem with enormous microbial diversity.[1,2] Molecular biological methods have allowed for a revolutionary advance in microbiological research: using these approaches, microbiological laboratories worldwide have begun to analyze the components of the human microbiota and to collaborate intensively in deciphering the human microbiome. It is noteworthy that the number of genes of our colonic microbiota exceeds the number of genes in the human genome by 150 times.[3] There are more than 50 bacterial phyla on Earth, but human gut-associated microbiota are dominated by four main phyla: *Firmicutes*, *Bacteroidetes*, *Actinobacteria* and *Proteobacteria*.[2] Fundamental comparative studies of human fecal microbiota have revealed the astonishing fact that each of us has a unique microbiota (i.e., there are considerable differences between the compositions of the microbiota of individuals). It has also been shown that the main bacterial populations comprising our microbiota stabilize during the first years of life. During this time, the microbiota develops and subsequently remains stable throughout our life in terms of the major bacterial populations, even after antibiotic treatments.[4] This large microbiome could produce an enormous quantity of molecules able to interact with the host; however, the role of these molecules remains to be elucidated. The existence of bacteria in the large intestine and their fundamental functions in nutrition and metabolism (fermentation of nondegradable oligosaccharides, metabolism of xenobiotics and activation or destruction of mutagenic metabolites) make the colonic microbiota a large fermentative organ.[5]

Metagenomic approaches have recently been used to demonstrate that the main functions of the small intestine microbiota may differ from the function of the colonic microbiota. The microbiota present in the small intestine is enriched in pathways and functions related to carbohydrate uptake and metabolism.[6] The small intestine contains the majority of immune cells in the body and is substantially involved in an appropriately functioning immune system; the small intestinal microbiota thus could play a more important role in development and maintenance of mucosal and systemic homeostasis. Dietary interventions and the administration of probiotics could be effective means of changing the composition of the relatively simple microbial community present in the small intestine and could thereby substantially affect this community's metabolic and immunomodulatory functions.

Although molecular biological analysis of the microbiota is providing new knowledge daily, functional studies concentrating on intensive analysis of the effects of the microbiota on the macroorganism are proceeding in parallel. The use of gnotobiological methods on experimental animals are an indispensable methodological tool in the study of the biological importance of the microbiota and the consequences of bacterial colonization. In these methods, mice or other species are bred by a complex technology in a sterile environment (i.e., free of live bacteria) in isolators and can then be colonized in a controlled way with defined strains of bacteria (including nonculturable species), and the effects of this colonization can be followed on both genetic and protein levels.[7,8]

MUCOSAL BARRIER FUNCTION

Epithelial surfaces have evolved protective mechanisms to resist microorganism invasion. Both mucosa and skin mediate contact between the organism and its external environment and there the organism encounters many antigenic, mitogenic and toxic stimuli present in food, normal microbiota and air. Moreover, most 'exogenous' pathogenic infections enter their host by the mucosal route. The mucosa and the internal environment of the organism are protected by the effective innate and adaptive immune systems. Almost 80% of the immunologically active cells of the body belong to the mucosal-associated immune system. The majority of these cells are present in tissues of the gastrointestinal tract, where the prevalence of immunogenic agents, including food and components of the microbiota, is the highest. Under physiological conditions, the gut is covered by the largest epithelial surface in the body (around 200 m^2 in humans), and it contains complex and poorly understood cell interactions that regulate responses to food antigens and to antigens of the normal bacterial flora.[9–15]

The barrier function of mucosal surfaces, particularly those of the intestine, is ensured by complex mechanisms acting on several levels. The microbiota itself forms an integral part of the natural mechanisms of mucosal surfaces and skin that safeguard the organism against pathogenic microorganisms. When the microbiota has an optimal composition, it prevents attachment and multiplication of pathogenic or virulent microorganisms on these surfaces and the invasion of these microorganisms into epithelial cells and the circulation. The intestinal microbiota plays an important role in pathogen resistance, both by direct interaction with pathogenic bacteria and by influencing the immune system.[16,17]

Mucins (highly glycosylated macromolecules) form the first barrier between the gut contents and epithelial cells, protecting them from direct contact with commensal bacteria and their components. Changes in the amount and/or the composition of mucus may lead to inflammatory responses.[18] The epithelial layer, which is covered by glycocalyx, forms a major barrier between the host and the environment. The epithelial layer of most mucosal surfaces consists of a single layer of interconnected, polarized epithelial cells. The epithelial layer of the gut mucosa is reinforced by junctions (tight junctions, adherens junctions and desmosomes) in the paracellular spaces between epithelial cells and forms an interconnected network. Tight junctions have been shown to act as a dynamic and strictly regulated port of entry that open and close in response to various signals such as cytokines and bacterial components, originating in the lumen, lamina propria and epithelium. Tight junctions participate in preserving cellular polarity and are regarded as key elements of intestinal diffusion mechanisms. The molecules forming tight junctions are connected to the cytoskeleton of epithelial cells and thus participate in determining the shape and structure of epithelial cells.[19] Epithelial cells participating in mucosal barrier function are conventional enterocytes (colonocytes in colon); goblet cells producing both mucus and trefoil peptides required for epithelial growth and repair; enteroendocrine cells producing neuroendocrine molecules having a paracrine effect; and Paneth cells secreting the antimicrobial peptides defensins. Neuropeptides, the products of the nervous system, are capable of increasing the permeability of tight junctions to macromolecules, thus modifying mucosal barrier function.[20]

The participation of innate immune factors in mucosal barrier function during the interaction with commensal microorganisms is now beginning to be appreciated. In addition to the well-known humoral components of innate immunity such as complement, lysozyme, lactoferrin and mannan-binding protein, other recently described factors have been intensively studied. An important humoral component of these non-specific innate mechanisms are the antimicrobial

peptides called defensins, widely distributed throughout the plant and animal kingdoms. Multiple types of these peptides are produced by Paneth cells, specialized cells present in the crypts of gut mucosa, and by other epithelial cells. In general, innate immune mechanisms are affected mainly by phagocytic cells (macrophages, neutrophils and dendritic cells) that can produce cytokines essential for inflammatory reactions and factors critical for the subsequent initiation of adaptive immunity. These cells initiate innate immune responses to microbes *via* the sensors called pattern recognition receptors.[21] These sensing structures, the Toll-like receptors, C-type lectin receptors, RIG-I-like receptors and nucleotide-binding domain and leucine-rich repeat containing proteins, sense pathogen motifs and transmit activation signals to their target cells. In addition to their strategic localization and absorptive, digestive and secretory functions, intestinal epithelial cells are equipped with various receptors to enable their participation in immunological processes. To prevent uncontrolled inflammatory responses to components of commensal microorganisms, the signaling pathways of these cells are tightly regulated by multiple molecules and pathways to ensure negative feedback mechanisms, similar to other mucosal innate immune cells.[16,22,23] We have previously shown that expression of the NOD2 molecule in the gut mucosa is affected by the presence of microbiota and that NOD2 expression influences the microbiota as well. The host *via* NOD2 and the intestinal commensal bacterial flora thus maintain homeostasis by regulating each other through feedback mechanisms.[24]

The main cells that present antigen to the adaptive arm of the mucosal immune system are dendritic cells.[25] Induction of tolerance or stimulation of a mucosal immune response depends on the participation of different populations of dendritic cells responsible for the activation of regulatory T-cell subpopulations.[26] Production of IL-10 and transforming growth factor-beta leads to the activation of regulatory T cells that inhibit the immune response and induce mucosal tolerance.[27] Pathogenic microorganisms induce the maturation of dendritic cells that lead to the activation of effector T cells indispensable for clearing infections and for the prevention of subsequent infection with the same or related pathogens.[25]

One of the main humoral defense mechanisms ensuring the barrier function of mucosal surfaces produced by the adaptive arm of the mucosal immune system is secretory immunoglobulin A (IgA). Polymeric secretory IgA is more resistant to proteolysis than other antibodies. Its primary task is to prevent both the adherence of bacteria to mucosal surfaces and the penetration of antigens into the internal environment of the organism. This is achieved by specific or nonspecific (using a reaction resembling lectin binding) mechanisms.[28–30] In individuals with selective IgA deficiency, the mucosal barrier is insufficient and is more permeable to allergens and other immunogens. Secretory IgA can react with some bactericidal substances contained in mucosal secretions (lactoperoxidase and lactoferrin) and transport them to bacterial surfaces.[28]

Mucosally administered antigens induce an immune response that is detectable not only locally, but also in circulation and in remote mucosal surfaces and exocrine glands.[31] Cells originating from organized mucosal lymphoid tissue migrate through lymph and blood after activation and home to mucosal surfaces and exocrine glands.[10,11,30] An example of the effect of migration and selective colonization by cells from the intestinal mucosal surfaces is the composition of the secretion from mammary glands: mother's milk. Apart from the nutritive components, mother's milk contains a number of immunologically nonspecific and specific factors and a large quantity of cells. These components protect the not yet completely developed intestine of the infant against infectious agents. The mammary gland is colonized by immune cells from the intestine of the enteromammary axis.[32] As a consequence of this colonization, the mammary secretions contain IgA antibodies and cells directed against antigens present in the maternal intestine, protecting the breastfed infant from threats present in its environment. This may involve bacteria from the maternal microbiota that colonize the intestine of the infant within the first 3 days of life and may have a pathological impact on the incompletely matured mucosa, as occurs in the case of necrotic enterocolitis. Therefore, the local protection provided for the infant's intestine by a number of molecules with immunomodulatory properties present in colostrum and milk, as well as maternal secretory antibody, is of major importance.[33]

THE ROLE OF THE MICROBIOTA IN POSTNATAL DEVELOPMENT OF INNATE AND ADAPTIVE IMMUNITY AND THE MUCOSAL BARRIER

The close symbiosis of the microbiota and human or animal hosts is the result of long evolution and mutual adaptation of both partners, which defines our ability to adapt to the ambient environment and defend ourselves against threats. The period in which the human host is most acutely influenced by the microbiota is the postnatal period, during which the germ-free neonate moves from the sterile environment of its mother's uterus into a world full of microorganisms and during which the neonate's mucosal and skin surfaces become gradually colonized. The composition of main bacterial populations does not stabilize until after the first few years of life. In this period, the microbiota gradually colonize the mucosal and skin surfaces of the neonate and exert the greatest effect on the development of the immune system.[34] The mode of neonate delivery is particularly important because infants delivered by caesarean section lack the first input of maternal bacteria, and their intestinal microbiota differ substantially.

Components of the intestinal microbiota play a crucial role in the postnatal development of the immune system. During the early postnatal period, the intestinal microbiota stimulates the development of both local and systemic immunity, while later on these components evoke inhibitory regulatory mechanisms intended to keep both mucosal and systemic immunity in check.[35–37]

The importance of the microbiota in the structural and functional features of the developing immune system was predicted by Professor Jaroslav Šterzl, who established the Laboratory of Gnotobiology at the Institute of Microbiology more than 50 years ago. This crucial development provided tools to study basic questions about the host–microbiota interaction using various animal models.[38–44] We have shown that microbial colonization of animals living in germ-free conditions results in an increase in immunoglobulin levels, the production of specific antibodies, substantial changes in mucosal-associated lymphocyte tissues and cell populations, changes in migration patterns and increases in the systemic immunological capacity.[35,40,42,43] In the early postnatal period, components of the normal microbiota induce a transient physiological inflammatory response in the gut associated with enlargement of the mucosal-associated lymphatic tissue and increases in its cellularity.[39,45]

The effect of microbial colonization on innate immune cells has been documented in our studies on the development of phagocytes, dendritic cells and intestinal epithelial cells.[24,46] Interestingly, the T-cell receptor repertoire is also influenced by colonization with microorganisms.[47] Recently we have studied the effect of the microbiota on the development of lymphatic subpopulations in BALB/c mice bred in

germ-free isolators or under conventional conditions and fed with sterile diets differing in contamination with microbial components. This study of lymphocyte subpopulations showed the mesenteric lymph nodes and Peyer's patches of germ-free mice fed by diets with lower lipopolysaccharide content contained fewer $CD4^+$ T lymphocytes than did secondary lymphoid organs from mice housed under conventional conditions. Germ-free mice kept on a diet with a high content of nonliving microbial components had more $CD4^+$ lymphocytes than animals kept on a diet with a low content of bacterial components. An important finding was that the development of regulatory ($CD4^+$ $FoxP3^+$) T lymphocytes depends on the presence of the microbiota and bacterial components in the diet: germ-free mice on a diet containing small amounts of lipopolysaccharide had fewer regulatory T lymphocytes.[45]

Interestingly, the microbial colonization of germ-free mice also speeds up the biochemical maturation of enterocytes, resulting in a shift in the specific activities of brush-border enzymes nearly to the extent found in conventional mice.[48] Moreover, a similar introduction of microorganisms alters the synthesis of sugar chains in membrane-associated glycoproteins, which could influence the gut barrier function.[14,49,50]

PARTICIPATION OF COMMENSAL BACTERIA AND THEIR COMPONENTS IN THE DEVELOPMENT OF INFLAMMATORY, AUTOIMMUNE AND NEOPLASTIC DISEASES

While the major cause of death in the less developed world remains infectious disease, the major killers in the developed world are cardiovascular diseases and cancer. Moreover, the steadily increasing prevalence of chronic disorders, like allergy, arthritic diseases and other inflammatory and autoimmune diseases, is causing significant morbidity.[51,52] These disorders represent an important medical problem because they have a devastating impact on quality of life and require long-standing medical care.

The main characteristics of inflammatory and autoimmune diseases are tissue destruction and functional impairment caused by immunologically mediated mechanisms that are principally the same as those that function against pathogenic infections. Both living bacteria and their components and metabolites are clearly responsible for many of those immunomodulatory mechanisms.[53] Considerable work on autoimmune, inflammatory and neoplastic diseases is aimed at investigating the pathogenic role of environmental agents, including these microbial components.[54,55]

In some cases, impaired function of the intestinal barrier leads to an increase in antibodies directed against antigens present in the intestinal lumen. It was recently shown that the appearance of these antibodies or/and autoantibodies in individuals lacking clinical symptoms may have important predictive value for the development of inflammatory and autoimmune diseases.[56,57]

In the case of autoimmune diseases, considerable effort has been made to understand mechanisms leading to the loss of self-tolerance. Infections have often been considered to initiate the process in genetically predisposed individuals. One major hypothesis explaining how infectious components can cause autoimmune reactions is based on the concept of crossreactivity, also known as ''molecular mimicry'', the similarity between the epitopes of autoantigens and epitopes of harmless environmental antigens.[58,59] Infections may also trigger the development of autoimmunity through the inadequate activation of innate immune cells.[60] The adjuvant activity of microbial components may participate in the stimulation of antigen presenting cells such as dendritic cells that leads to the abnormal processing and presentation of self-antigens. Superantigens are microbial components that have been shown to be particularly effective in inducing inflammatory reactions.

Genome-wide association studies on large human cohorts are used to identify the role of genes mutated in chronic human diseases. These studies allow us to suggest not only the mechanisms but also the interacting environmental factors or infectious components involved in disease initiation and maintenance.[61,62]

Homeostasis of the intestinal mucosa may be disturbed by pathogenic microorganisms and toxins attacking the intestine or by inadequately functioning components of the immune system, as observed in immunodeficiency or in cases of dysregulated mechanisms of the mucosal immune system. The intestinal mucosa can be affected as a consequence of either insufficient activity or exaggerated activation of the immune system.[27,63] Various complex diseases may occur as a consequence of disturbances of mucosal barrier function or of changes in mechanisms regulating mucosal immunity to food or components of the microbiota.[64,65] Studies showing both interindividual differences and a disease-specific pattern in the composition of the microbiota in humans are of particular interest. Nevertheless, the complexity and interindividual variation of the gut microbiota composition in humans represents a confounding factor in the efforts to determine the possible significance of individual commensal microbial organisms in disease pathogenesis.

Patients often come to the clinic only after their disease has become symptomatic, making the understanding of the early events leading to disease difficult. Experimentally induced and spontaneously developing animal models of human diseases allow us to examine the role of genetic and environmental factors in early events during disease development, to elucidate the pathogenic mechanisms and to develop new preventive and therapeutic strategies, despite these models sometimes being too artificial to be comparable with human disease. Examples of diseases in which barrier dysfunction and involvement of the microbiota in human disease have been suggested and cases in which the use of germ-free or gnotobiotic animal models of disease were beneficial are listed below.

INFLAMMATORY BOWEL DISEASE (IBD)

Idiopathic IBD, Crohn's disease and ulcerative colitis are severe chronic disorders affecting approximately 0.2% of the human population. Despite intense study, the etiology and pathogenesis of these diseases remain unclear. The pathogenesis of IBD involves interactions among immune, environmental and genetic factors; the combination of these factors results in the induction of inflammation, subsequent development of mucosal lesions and repair. Disruption of T lymphocyte regulatory functions and impairment of the mucosal immune response to normal bacterial flora play a crucial role in the pathogenesis of chronic intestinal inflammation. This may implicate the loss of local physiological regulatory mechanisms and perhaps a breakdown of oral tolerance to luminal antigens in these diseases.[66–70] This suggests that the intestinal mucosa is one of the most sensitive indicators of immune dysfunction. The demonstration of abnormal T-cell responsiveness against indigenous microbiota in human IBD suggested that commensals may initiate and/or perpetuate the intestinal inflammation seen in IBD.[71] Recent results of genome-wide association studies performed in large cohorts of patients confirmed the previously suggested participation of microbial components in the development of Crohn's disease and ulcerative colitis.[72] Many of the mutations found were in genes encoding recognition, processing and killing of microorganisms and the regulation of immune processes.

Interestingly, some of these gene defects were also found in patients with other autoimmune diseases.

Several animal models of spontaneously developing intestinal inflammation suggest that innate immunity, mucosal barrier defects or disruption of T lymphocyte regulatory functions could lead to chronic intestinal inflammation. A number of genetically manipulated mice, such as mice deficient in IL-2 or IL-10, develop spontaneous chronic intestinal inflammation.[73] Interestingly, the disease can be prevented when these mice are reared in germ-free conditions.[74,75] Similarly, BALB/c mice develop a much milder form of acute dextran sulfate sodium induced colitis in germ-free conditions compared to conventionally reared mice.[76]

In our studies, we have used a T-cell transfer model of chronic colitis in germ-free and other gnotobiotic mice to elucidate the effects of colonization with defined mixtures of microbes on the development of intestinal inflammation. We observed that after the transfer of $CD4^{+}CD45RB^{high}$ T cells into severe combined immunodeficient mice, severe inflammation was present in mice colonized with a cocktail of specific pathogen-free microbiota along with segmented, filamentous bacteria. Interestingly, germ-free mice, mice treated with segmented, filamentous bacteria alone or mice treated with the specific pathogen-free cocktail did not exhibit markers of severe intestinal inflammation.[77]

Oral treatment with lysed bacteria may influence the development of experimentally induced intestinal inflammation. We have shown that the severity of dextran sulfate sodium-induced intestinal inflammation in BALB/c mice is reduced by oral administration of a sonicated microbiota containing anaerobic bacteria.[78] Furthermore, we found that this effect could be modulated *via* the manipulation of the gut microbiota and immunomodulation of the mucosal and the systemic immune response.[79] Thus, the mechanisms of this protective and therapeutic effect should be elucidated more precisely, and this novel approach may be used for the development of a potential vaccine.

CELIAC DISEASE

Celiac disease is a chronic immune-mediated enteropathy that is triggered by dietary wheat gluten or related prolamins in genetically susceptible individuals. It is characterized by an increase in the cellularity (intraepithelial lymphocytes) and atrophy of jejunal mucosa. The autoimmune nature of this disease was confirmed by the presence of autoimmune mechanisms directed against several autoantigens, including the most diagnostically important autoantigen, tissue transglutaminase. The frequent association between celiac disease and other autoimmune diseases, particularly type 1 diabetes (T1D) and thyroiditis, suggests that celiac enteropathy shares certain pathogenic mechanisms with other autoimmune diseases.[80] Indeed, gut mucosal barrier dysfunction was repeatedly demonstrated and confirmed by genetic studies in patients with celiac disease and T1D.[81–83] Several intestinal viral triggers including adenovirus, hepatitis C virus, and rotavirus and bacterial infections capable of initiating or augmenting gut mucosal responses to gluten were suggested to play a role in the pathogenic mechanism of this disease.[84] Abnormal components found among the microbial inhabitants adhering to the diseased jejunal mucosa have been described and recently analyzed using new microbiological methods by Ou *et al.*[85] Profound changes in the fecal and duodenal microbiota composition of patients with active disease who are on a gluten-free diet have also been demonstrated.[86] Interestingly, some commensal bacteria, such as *Escherichia coli*, promoted the activation of innate immune cells by gliadin, whereas others (*Bifidobacteria*) exerted inhibitory effects.[87]

There are a limited number of suitable animal models for this disease. Using long-term intragastric application of gluten to Wistar-AVN rats starting at birth, we were able to induce the main features of gluten enteropathy: an increase in intraepithelial lymphocytes, crypt hyperplasia and shortening of the villi in the jejunal mucosa. Moreover, we found similar changes in mucosal structures after transfer of intestinal lymphocytes into the intestinal loops of inbred germ-free recipients. Changes appearing after gluten, but not albumin, feeding were inducible in germ-free rats, i.e., in the absence of microbiota, suggesting the activation of intestinal immune cells by this unique food protein.[88]

T1D

Type 1 (insulin-dependent) diabetes mellitus is one of the most well-studied organ-specific autoimmune diseases. It develops as a consequence of selective destruction of pancreatic insulin-producing beta cells within the islets of Langerhans. Autoimmune reactions against beta cells may arise from activation of the immune system in genetically predisposed individuals by environmental factors that bear epitopes similar to those expressed by the beta cell. Several mechanisms such as molecular mimicry, metabolic stress on beta cells, cryptic epitope exposure and costimulatory molecule upregulation have been proposed but not fully validated in the pathogenesis of T1D. Recently, T1D has been considered a consequence of dysregulated or inadequately developed regulatory immune responses in genetically predisposed individuals, similar to other autoimmune diseases.

The rapid increase in the incidence of T1D in developed countries during recent decades points to the role of environmental factors in this disease. Candidate environmental factors influencing T1D include various microbial and food components encountered at mucosal surfaces as well as gut mucosal parameters such as gut permeability.[89] The main difficulty in characterizing the environmental factors and mechanisms in T1D and possibly other autoimmune diseases is their complexity, the long lag period between the induction or disease-modifying events and the clinical onset of the disease, and the lack of studies in environmentally-defined, gnotobiological animal models. Furthermore, environmental factors in T1D seem to prevent full penetration of the disease rather than trigger it.

In non-obese diabetic (NOD) mice and biobreeding rats, the two well-established animal models of spontaneously developing autoimmune diabetes, the quality of specific pathogen-free housing facilities influences incidence of the disease. Animal facilities with positively defined specific pathogen-free microbiota (e.g., altered Schaedler microflora), antibiotic decontamination or rederivation of the breeding nucleus facilitate high diabetes incidence. Thus, clean conditions increase T1D incidence, whereas infections, including parasite infections and immunization with bacterial components, decrease the incidence.[90] We have shown rapid disease onset and 100% diabetes incidence in NOD females reared in germ-free conditions.[91] Wen *et al.* have also recently reported high diabetes incidence in germ-free mice and have documented an involvement of innate immune mechanisms in the disease. These findings indicate that some not yet well-defined components of the commensal microbiota exert diabetes-protective effects.[92]

The course of T1D may also be influenced by food. In both biobreeding rats and NOD mice, the diabetes-promoting agents are not carbohydrates but come mainly from the plant protein fraction of natural diets.[93] We have documented that a gluten-free diet, but also a diet with high gluten content, highly decreases diabetes incidence in NOD mice.[94,95] In addition, different mechanisms are responsible for

the disease prevention: some diets have a microbiota-dependent diabetes-protective effect, whereas others prevent T1D in a microbiota-independent manner.[91] Gliadin, the component of wheat gluten that triggers celiac diseases in susceptible individuals, was shown to activate innate immune mechanisms and to increase intestinal permeability.[83,96,97]

Increased gut permeability preceding clinical onset of the disease and signs of activation of the gut immune system were described in children with T1D and were related to the pathogenesis of this disease.[89] An impaired gut barrier function and subsequent consequences on the development of the disease were also demonstrated in the biobreeding rat model of T1D.[98] Thus, apart from various environmental factors acting in T1D, parameters of the gut mucosa forming an interface between the self and the environment further contribute to the complexity of the disease.

NEUROLOGICAL AND PSYCHIATRIC DISEASES

One of the most frequent and severe autoimmune neurological diseases is the demyelinating disease multiple sclerosis. The disease affects mainly young people, finally leading to their invalidity. Changes in gut barrier function, i.e., increased intestinal permeability in patients, as well as in their relatives, has been reported.[99] Viruses and bacteria have been suggested to participate in the pathogenesis of multiple sclerosis. Based on morphological and immunological findings in the brains of patients, attention has recently been given to the common infection with Epstein–Barr virus.[100] Bacterial involvement in the pathogenesis of multiple sclerosis was suggested after the bacterial peptidoglycan was found within antigen presenting cells (dendritic cells and macrophages) in the brains of patients but not of control individuals.[101] The potential role of molecular mimicry associated with infections was studied by Westall.[58] By comparing the sequences from three known encephalitogenic peptides with all known human bacterial and viral agents, this group found that mimics are present in a wide variety of microorganisms. Interestingly, the mimics were present predominantly in non-pathogenic gut bacteria.

Demyelination can be experimentally induced and achieved by immunization of mice with autoantigenic molecules isolated from central nervous system. This widely used model of multiple sclerosis, experimental autoimmune encephalomyelitis, has been invaluable in elucidating the pathogenesis of this debilitating disease and in creating new therapeutic approaches.[102] The role of the components of the microbiota in the pathogenesis of disease using this experimental model has recently been documented by Kasper's group, and the results of this study have been used to propose a novel treatment.[103,104]

The gut–brain axis is a bidirectional communication system through which the brain modulates gastrointestinal function and through which the gastrointestinal system is monitored by the brain. Neural and immunological and endocrine mechanisms are involved in this communication. The intestinal microbiota influences the gastrointestinal physiology, including the development and function of enteric nervous system.[20,105] The enteric nervous system (the 'second brain') directly controls the gastrointestinal tract functions. It consists of more neurons than there are in spinal cord (about 10^8), organized in myenteric and submucosal plexuses.[106] Interestingly, recent findings suggest a potential involvement of these structures in frequently occurring neurodegenerative disorders, like Parkinson's disease. Characteristic Lewy bodies, pathological hallmarks of Parkinson's disease, were found in intestinal biopsies of patients with Parkinson's disease.[107]

There is increasing evidence that the immune system, inflammation and mucosal barrier function are involved in the pathogenesis of some psychiatric diseases. Autism is an important mental illness and has attracted the attention of researchers due to its sharply increasing incidence in developed countries. Changes in antigenic load due to the impairment of gut barrier function were recently suggested as a triggering factor.[108] Autistic enterocolitis and changes in intestinal permeability were described in this early onset developmental disorder.[109] Moreover, urinary metabolic phenotyping has determined biochemical changes that were consistent with abnormalities in the composition of the gut microbiota found in autistic children.[110,111] Interestingly, in another mental illness, depression, 'leaky gut' has been suggested to play a pathogenic role: this assumption was based on findings of altered intestinal permeability in patients and their first-degree relatives.[112]

Analysis of behavioral changes in experimental animal models of neuropsychiatric diseases has started to be used to elucidate the role of the mucosal barrier function and the involvement of environmental factors in disease pathogenesis.[113] We have studied the behavioral changes occurring after induction of intestinal mucosal changes resembling celiac diseases by feeding of high doses of gluten to rats, and we found a higher emotionality in an open field test.[114] It is interesting to note that behavioral and psychological changes are often present in patients with active celiac disease, which is associated with findings of regional cerebral hypoperfusion in their brains.[115]

RHEUMATIC DISEASES

The involvement of intestinal changes in the pathogenic mechanisms of rheumatic diseases was suggested by findings of increased intestinal permeability and the presence of gastrointestinal symptoms in patients with juvenile idiopathic arthritis.[116] The frequent occurrence of arthritis in patients suffering from IBD suggests participation of the gut in this immune mediated rheumatic disorder.[117]

Infection with intestinal microbial pathogens such as *Salmonella*, *Shigella* and *Yersinia* precedes the development of reactive arthritis; these infections can trigger autoimmune reactions in joints.[118] Moreover, increased level of antibodies directed against antigens of certain species of gut bacteria (e.g. *Proteus*) suggests that there is an pathogenic relationship between these bacteria and rheumatoid arthritis.[119] Similarly, increased titers of anti-*Klebsiella* antibodies in patients with ankylosing spondylitis suggest that infection with this bacterium could be a triggering factor in these patients.[120] Only recently has the involvement of the gut microbiota community in the pathogenesis of rheumatic diseases been properly analyzed. Most studies involving the gut microbiota composition in rheumatoid arthritis have been performed using classical cultivation methods that do not allow analysis of the non-cultivable majority of gut microbiota. Studies based on the use of new molecular biological methods demonstrating alteration of the gut microbiota composition in patients with rheumatic diseases (e.g., juvenile arthritis) have appeared only recently.[121]

Animal models of rheumatic disease are frequently used, as in other diseases, to study pathogenic mechanisms and to develop new therapeutic approaches. Currently, these models are being used to study the participation of gut microbiota in disease development.[122] The rat HLA-B27 transgenic model of ankylosing spondylitis spontaneously develops this disease, associated with colitis, when reared in conventional conditions (i.e., with microbiota). After transfer into germ-free conditions, the transgenic rats lose inflammatory changes in the gut as well as in joints.[123] Alleviation of symptoms and inflammatory

changes through oral application of probiotics have been described in an experimental model of adjuvant-induced arthritis.[124] Exciting results were obtained from a recent study performed using a mouse model of rheumatoid arthritis, where it was demonstrated that a germ-free state decreases the clinical and autoimmune markers of arthritis. However, colonization with a unique non-cultivable bacterial strain belonging to mouse commensals, i.e., with segmented filamentous bacteria, induced the Th17 subpopulation, leading to clinical symptoms and increases in autoantibody production.[125]

OBESITY, CARDIOVASCULAR DISEASES AND ATHEROSCLEROSIS

In addition to the well-known role of intestinal bacteria in nutrition, commensal bacteria were found to play an important role in many physiological processes. Considerable interest in this role was generated by the findings of Jeffrey Gordon concerning changes in the expression of genes in germ-free mice following colonization by certain strains of intestinal bacteria.[7] These studies demonstrated significant effects of bacterial colonization on the expression of a wide range of genes, some of which are involved in metabolism.[50] The study from Backhed *et al.* examined the relationship between the composition of the microbiota and obesity.[126] Experimental models of genetically obese mice (leptin deficient ob/ob mice) and gnotobiological techniques (germ-free mice) were used in this study. These experiments demonstrated that the colonization of the intestine of germ-free mice by microbes from conventional mice led to a 40% increase in body fat over a relatively short period of time (2 weeks), despite the maintenance of low food intake. In other experiments, germ-free mice were colonized with the microbiota of obese mice and a control slim strain. Colonization with microbiota from obese mice induced a higher rise in body fat than did colonization with the microbiota from slim mice. The composition of the intestinal bacteria of the obese leptin deficient mice when analyzed by molecular biological methods was found to differ from that of the slim mice, particularly concerning the proportion of the two bacterial groups *Firmicutes* and *Bacteroidetes*: obese mice exhibited a 50% lower frequency of *Bacteroidetes* and an increased proportion of *Firmicutes*. These changes in the microbiota composition increased the ability to break down fiber into short chain fatty acids and to release additional energy that could be stored as fat.[126,127]

Interesting data have been generated in analyses of human microbiota. These results confirmed the data obtained in mice: obese patients had a lower proportion of *Bacteroidetes* and, if they lost weight during a year, the proportion of *Firmicutes* in their intestinal microbiota was comparable with that found in slim persons.[128] The recent study from Backhed *et al.* demonstrated that the colonization of germ-free mice leads to an increased *de novo* production of fat. This phenomenon was associated with lowered expression of the intestinal factor Fiaf, which takes part in the regulation of fat production.[126]

Many laboratories have endeavored to analyze the mechanisms by which intestinal bacteria affect the use of energy from food and to try to find bacterial strains whose administration would aid in the treatment of obesity, which puts the health of millions of people at risk for developing cardiovascular and other diseases.

Infections with *Chlamydia*, *Helicobacter pylori* or periodontopathic bacteria have been considered to increase the risk of development of cardiovascular disease.[52,129] Interest in the participation of the gut and its microbes was highlighted by findings demonstrating altered intestinal function, including increased permeability and augmented bacterial biofilm in patients with chronic heart failure.[130]

Experimental studies concentrated on the use of an advantageous mouse model of atherosclerosis, apolipoprotein E-deficient mice ($ApoE^{-/-}$). It has been demonstrated that infectious stimuli are not needed for the development of atherosclerotic plaques in ApoE-deficient mice fed by a high-cholesterol diet.[131] We also used this model, and have observed that, in contrast to the absence of atherosclerotic plaques in conventionally reared ApoE-deficient mice, germ-free ApoE-deficient mice consuming the same low-cholesterol standard diet exhibited developed atherosclerotic plaques in the aorta. Differences in the atherosclerotic plaques between germ-free and ApoE-deficient mice containing microbiota are not as apparent when the mice are fed by a high-cholesterol diet. These results document the protective effect of the microbiota on atherosclerosis development.[132]

ALLERGY

Epidemiological increases in the incidence of allergy, the most common chronic inflammatory disease, have occurred in recent years in economically developed countries and have triggered interest in potential environmental factors.[51,133] The search for an explanation for this trend resulted in the hypothesis that exaggerated hygienic conditions in these countries have decreased the quantity of natural infectious stimuli from the external environment, disturbing the well-balanced development of subpopulations of T cells, particularly the subpopulation of regulatory T cells ('hygiene hypothesis').[53,134,135] Recent microbiological analyses performed using classical and molecular biological techniques have demonstrated differences in the composition of intestinal microbiota between children from highly developed and underdeveloped countries. The former are born under controlled conditions in hospitals with maximal care and observance of hygienic measures. Consequently, the spectrum of microbes of their intestinal tract is much narrower than that of children from less developed countries.[34] Unfortunately, there is limited understanding of the role of intestinal lymphatic tissues and mucosal immunity in these processes. Recent studies have documented the changes in fecal microbiota in children suffering from food allergy.[136] Many efforts to influence the microbiotal composition of children in the early postnatal period have been attempted by the application of probiotics, and the results measured by allergy incidence later in life are promising.[137–139]

There are few experimental studies concerned with whether and how microbiota influences the development of allergy.[140] We have addressed the question of whether the intestinal microbiota affects the induction of mucosal (oral) tolerance against the birch pollen allergen. The Bet v1 allergen was applied intranasally or intragastrically in an experimental model of allergy induced by subcutaneous sensitization with the same allergen, and induction of tolerance was tested after application of an inducing allergen dose. Mice reared in germ-free and conventional environments did not differ in their ability to induce tolerance *via* the mucosal route or in their ability to induce a Th2 response. Therefore, we have demonstrated that the ability to induce mucosal tolerance is independent of the presence of microbiota in this model.[141]

CANCER

The involvement of infectious causes in the etiology of cancer has attracted the attention of researchers in recent years. At present, an association of cancer with bacterial and viral infectious agents is found in approximately 20% of all malignancies.[142] This is due to the increasing number of studies demonstrating the role of inflammation in establishing conditions that can deeply alter local immune responses

and, consequently, tissue homeostasis. In particular, inflammatory mediators such as IL-1, tumor-necrosis factor-α, IL-8, nitric oxide or prostaglandin-2 derivatives and molecules of the inflammatory pathways have been shown to be involved in a progressive interplay between immune cells and cells of a tissue undergoing transformation.[143] This association between inflammation and cancer has been highlighted in studies of IBD. The degree and prolongation of the duration of ulcerative colitis were recognized as factors leading to increased risk of gastrointestinal cancer development.[144,145] A correlation between gut microbiota composition and gastrointestinal cancers was examined in experimental animal models and clinical/epidemiological studies of environmental etiological factors. An association between a Western-style diet (red meat, fats and low vegetable intake) and changes in the composition of the gut microbiota has been observed in animal and human studies. This was linked to increased activities of fecal bacterial enzymes, as well as modification of sulfidogenesis and biliary acid metabolism with an impact on development of procarcinogenic conditions.[146,147] Interestingly, microbiota products can influence not only the local intestinal environment but also distant organs. Gut microbiota can metabolize certain plant-derived foods into biologically active compounds, e.g., enterolignans, that may play a role in carcinogenesis.[148] A recent meta-analysis indicated that these phytohormones may decrease the incidence of breast cancer.[149]

The highest production of carcinogens was associated with gut anaerobic bacteria and was lowered by supplementation with live lactobacilli.[150] *H. pylori* infection of the gastric mucosa was shown to create the conditions for developing ulcers, adenocarcinomas and gastric B-cell lymphomas. In fact, continuous inflammation induced by the bacteria activates cellular pathways inducing changes in mucin production (MUC2), as well as metaplasia and proliferation. Changes in mucin production and structure have been described both in gastric neoplastic conditions related to *H. pylori* and during the development of colorectal carcinoma.[151] These alterations progressively modify the relationship between the microbiota and the mucosal epithelia due to changes in the adhesiveness and integrity of the mucosal barrier. Some bacteria are able to induce modification of mucosal permeability, facilitating the translocation of bacteria and bacterial toxins (e.g., lipopolysaccharide). The inflammatory responses elicited were demonstrated to be able to enhance cancer progression.[152]

Bacteria represent a continuous stimulus for maintaining activated immunity in the gut mucosa and actively participate in the metabolism of bile and food components. Since germ-free mice lack this stimulus, they serve as a useful tool to study the role of bacteria in intestinal carcinogenesis. Compared with conventional mice, the incidence of both spontaneous and induced tumors is significantly lower in germ-free conditions.[153,154] Our studies of the participation of the microbiota in carcinogenetic processes were performed in the rat model of colorectal carcinoma and stressed the importance of the intestinal environment on the modulation of antitumor immunity. Compared to conventionally reared animals, germ-free rats develop fewer and smaller tumors. This result was associated with more active local and systemic immune responses.[155]

PROBIOTICS AND PREBIOTICS IN DISEASE PREVENTION AND THERAPY

Increased interest in the effects of the intestinal microbiota on human health has resulted in attempts to optimize the composition of the microbiota by dietary interventions or with probiotics, prebiotics or both (symbiotics).[156] The effects of probiotics depend on the properties of the microorganism used with both species- and strain-specific effects. Probiotics are mainly lactic acid bacteria (*Lactobacilli* and *Bifidobacteria*), but other bacterial species (enterococci or some strains of *E. coli*) and yeast have also been used as probiotics. Probiotic bacteria are often consumed in foods such as yogurts and cheese, in food supplements, or as drugs. Prebiotics are compounds that support the proliferation of beneficial bacteria (*Lactobacilli* and *Bifidobacteria*) in the intestine and include some saccharides (e.g., inulin). Probiotics have been shown to favorably influence the development and stability of the microbiota, inhibit the colonization by pathogens, influence the mucosal barrier by trophic effects on the intestinal epithelium, protect against physiological stress, and stimulate both specific and nonspecific components of the immune system.[139,157–163] Probiotics may well replace antibiotics whose resistance has been steadily increasing. Similar to the effects of microbiota, the effects of administration of probiotics and prebiotics are being intensively studied. Experiments using cell cultures and animal models are being performed to demonstrate the anti-inflammatory and immunomodulatory effects of different strains of probiotic microorganisms. To date, well-controlled clinical studies to clearly document the therapeutic or preventive effects of probiotics in various diseases are scarce. Even so, the therapeutic or preventive effects of certain probiotics have been documented in therapy of pouchitis, traveler's and antibiotic-associated diarrhea, irritable bowel syndrome and rotavirus enteritis.[164,165] The effects of probiotics in allergy prevention and therapy have been intensively studied, but the results are not yet conclusive.[166] A long-lasting effect on allergy prevention has been demonstrated in some studies.[137] The effects of probiotics on autoimmune and neoplastic diseases have been studied far less than the effects on allergy and intestinal diseases.[167,168]

Molecular mechanisms of probiotic effects in the intestine have begun to be elucidated in humans by analyzing local changes in their transcriptome.[169] The use of recombinant probiotic bacteria expressing a number of interesting biologically active molecules, such as allergens that could induce tolerance and inhibit allergic responses after administration in the gut, represents an exciting new direction.[170,171]

CONCLUSION

Just as homeostasis of our body systems is the product of many complex, redundant mechanisms, multigenic disease development is also dependent on both missing and overactivated pathways. The goal to find a common factor in the disease pathogenesis is difficult, genetic and pathophysiological data are incomplete, and the individual variability is enormous. Examination of the role of the microbiota in human illnesses using animal models of human diseases reared in defined (gnotobiotic) conditions could allow insight into the unusual complexity of the mechanisms involved in the initiation and maintenance of chronic diseases. Although the most important findings in this fascinating field are still to come, it is clear that our bacterial companions affect our fates more than previously assumed.

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