The interaction between the mucosal immune system and the commensal microflora of the colon
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Introduction and outline of the thesis

Introduction
Crohn's disease and ulcerative colitis are chronic inflammatory diseases of the gastrointestinal tract. They are collectively named as Inflammatory Bowel Diseases (IBD). The prevalence of Crohn's disease and ulcerative colitis is both about 50/100.000 (together about 1/1000). They are identified and diagnosed by the appearance of a set of clinical, endoscopic, and histologic characteristics. In ulcerative colitis the inflammatory response is largely restricted to the mucosa and submucosa, but in Crohn's disease inflammation extends through the intestinal wall from mucosa to serosa. Ulcerative colitis is restricted to the colon and, and colectomy is curative. In contrast, Crohn's disease has the potential to involve the patient's entire gastrointestinal tract, but is mainly present in the colon and terminal ileum. Resection of the inflamed segment is not curative in Crohn's disease, and inflammation is likely to recur proximal of the anastomosis. The inflammation in ulcerative colitis is continuous from the anus to the proximal margin, whereas, in Crohn's disease normal mucosa may be present between affected areas (skip lesions) 1. Smoking is known to cause exacerbations in Crohn's disease. In contrast, in ulcerative colitis smoking may attenuate manifestations of disease 1,2. Despite these differences, there is a group of patients whose clinical picture falls between the two diseases: these patients have indeterminate colitis.

The etiology and pathogenesis of ulcerative colitis and Crohn's disease are not known. Presumably, both diseases have a multifactorial origin in which several genes, environmental factors, a dysregulated immune response, a disturbed barrier between intestinal lumen and mucosal immune system, and the commensal microflora may be important 1.

Genes in the pathogenesis of IBD
In siblings and offspring of IBD patients there is a 7% lifetime risk of developing IBD 3. Monozygotic twins are even 45% concordant for Crohn's disease 1. This suggests a genetic factor or genetic factors. Variants of the CARD15-gene have been identified to have a strong association with Crohn's disease. The product of the CARD15-gene is located within macrophages, has a binding site for bacterial lipopolysaccharide and perhaps plays a role in regulating nuclear factor-kappa B activation and macrophage apoptosis 4. Persons who are homozygous for variant CARD15 may have a 20-fold or more increase in susceptibility to Crohn's disease, with a particular predilection for ileal disease. Only 4% of patients with Crohn's disease are homozygous for these CARD15 variants opposed to 0.02% in controls and patients with ulcerative colitis 1.

Environmental factors in the pathogenesis of IBD
The incidence and prevalence of Crohn's disease and ulcerative colitis vary greatly in different geographic locations. The highest prevalence rates are in northern Europe and North America, whereas very low rates are found in Africa, Asia and South America 1. Although genetic factors may explain some of these differences, environmental factors may also be important. Smoking, as mentioned above, is a strong environmental factor affecting both ulcerative colitis and Crohn's disease albeit in opposing ways 1. Also the intestinal microflora can be considered as an environmental factor that most probably plays a role in the pathogenesis of Crohn's disease. This is discussed below.
Mucosal immune response in the pathogenesis of IBD
Clear evidence exist for activation of the mucosal immune response in IBD. The lamina propria is infiltrated with lymphocytes and macrophages and there is a 10 fold increase in the amount of plasma cells with a shift towards IgG and IgM producing plasma cells in the inflamed mucosa. Also the production of proinflammatory cytokines (IL-1, IL-6, TNF-alpha) is increased within the inflamed mucosa. The mucosa of patients with Crohn's disease is dominated by CD4+ lymphocytes with a type 1 helper-T-cell (Th1) phenotype, characterised by the production of interferon-gamma and interleukin-2. In contrast, the mucosa in patients with ulcerative colitis is dominated by CD4+ lymphocytes with a type 2 helper-T-cell phenotype (Th2), characterised by the production of TGF-beta and interleukin-5. Presumably Th1 cytokines activate macrophages, which in turn, produce interleukin-12 and interleukin-18, tumor necrosis factor (TNF), interleukin-1, and interleukin-6.

An immune response is either initiated by a specific antigen (a normal immune response to an antigen), or by a loss of factors that normally suppress or preclude an immune response (an abnormal immune response to an ‘autoantigen’).

Prevention of mucosal immune reactivity
In healthy individuals, a low-grade chronic inflammation is present in the intestinal mucosa. Presumably, this chronic inflammation is a product of chronic exposure of the mucosa to luminal antigens, as evidenced by the absence of IgA secreting plasma cells in the gut lamina propria of germfree and antigen free mice. Failure to suppress this immune response could result in an uncontrolled mucosal immune activation as seen in IBD.

Apart from the epithelial-mucus barrier there are powerful mechanisms that prevent mucosal inflammation: (a) phagocytosis of bacteria that have penetrated into the mucosa and (b) mucosal immunological tolerance for the antigens of commensal bacteria that have penetrated into the mucosa.

Findings by Duchmann et al. suggest that there exists some sort of mucosal tolerance for an individual's own microflora. Their data show that mucosal T cells, isolated from the colon of healthy individuals, do not proliferate in response to commensal colonic bacteria derived from the same individual whereas these T cells proliferate in the presence of bacteria derived from another individual. In marked contrast, however, mucosal T cells from IBD patients with active disease proliferate intensely when incubated with commensal bacteria derived from the same patient. This suggests that the normal mucosal tolerance may be disrupted in IBD patients. How tolerance at mucosal sites is regulated is not exactly known, but it is clear that many different cells, cytokines and other factors are involved. Current theories suggest a role for the so-called mucosal regulatory Tr1 cells in maintaining tolerance. Tr1 cells are antigen-specific mucosal CD4+ T cells that produce large amounts of IL-10 in response to antigen recognition. IL-10 may suppress activation of other T cells in the local environment thereby inducing a more general mucosal tolerance including the humoral immune response.

The epithelial-mucus barrier in IBD
In healthy individuals, a single layer of epithelial cells and mucus separates the abundant luminal antigens from an extensive mucosal immune system. It is not known whether the gelatinous thick mucus layer that contains large amounts of dimeric IgA precludes direct contact between commensal bacteria and epithelial cells. A temporary loss of mucosal barrier function (resulting in an influx of commensal bacteria and their antigens into the mucosa) due
to bacterial or viral enteric infections, NSAIDs or hypersensitivity reactions to food antigens, may precipitate exacerbations of Crohn's disease in susceptible patients. On the other hand, anticancer therapy in non-IBD patients may lead to extensive mucosal sloughing, yet this heals within days without causing a chronic colitis.

**Intestinal microflora in the pathogenesis of IBD**

The human intestinal tract harbors a complex microbial ecosystem, usually referred to as the normal commensal microflora (see below). Circumstantial evidence suggests that the commensal intestinal bacterial flora may play a role in the pathogenesis of IBD. This conclusion is based on the following arguments: (i) IBD occurs most frequently in intestinal regions colonised by the highest bacterial concentrations (colon and terminal ileum); (ii) Crohn's disease (but not ulcerative colitis) may improve when luminal bacterial concentrations are reduced by elemental diets, split ileostomy, intestinal lavage or broad spectrum antibiotics; (iii) in Crohn's disease rechallenge of an excluded colon or neoterminal ileum with autologous intestinal contents results in mucosal inflammation; (iv) there are several animal models (for instance mice with a targeted deletion for T cell receptor alpha, IL-2 or IL-10 and rats transgenic for HLA-B27) that develop colitis when they are maintained under conventional conditions but not under germfree conditions. Collectively, these data suggest that the presence of indigenous bacteria is required for the induction of intestinal inflammation in Crohn's disease and possibly also in ulcerative colitis. Presumably, the combination of (a) an inappropriate mucosal immune response, (b) a reduction of the epithelial-mucus barrier and (c) large amounts of bacterial antigens may be responsible for the initiation and perpetuation of the chronic inflammation.

**Normal commensal intestinal microflora**

The normal commensal microflora consists mainly (>99.9%) of obligatory anaerobic bacteria. The highest bacterial concentrations are found in the colon and terminal ileum: 10^{10} - 10^{12} bacteria per gram intestinal content. The normal microflora has many beneficial functions: synthesis of vitamin K and growth factors for host intestinal cells, prevention of outgrowth of potentially pathogenic bacteria (colonization resistance), stimulation of intestinal motility, promotion of the enterohepatic circulation by deconjugation of bile acids, stimulation and maturation induction of the gut immune system and modulation of the expression of a number of epithelial genes. Each individual has a characteristic commensal colon microflora that is relatively stable over time. Little is known about the composition of the microflora and possible differences in composition between the different colon regions, terminal ileum and faeces.

In the last ten years a new method has been developed to study bacteria without culturing: Fluorescence In Situ Hybridisation (FISH). FISH has proven to be a very powerful technique for the study of fecal anaerobic bacteria. All bacteria have 10,000 - 100,000 copies of ribosomal 16S rRNA which contains conserved regions (i.e. conserved for all bacteria), variable regions (i.e. conserved for each group of bacteria) and hypervariable regions (i.e. typical for specific bacterial strains). In this way 16S rRNA-targeted oligonucleotide probes can be constructed with specificity for all bacteria (BACT338), certain groups of bacteria (for instance BIF164 which specifically hybridises with Bifidobacteria) or specific bacterial strains. If a fluorescent label is attached to these probes, hybridised bacteria can be visualised under a fluorescent microscope or with flow cytometry: this is called FISH.

**Intestinal commensal microflora and its interaction with the host**

Because of the putative role of the intestinal microflora in the pathogenesis of IBD there is a
Chapter 1

growing interest in the interaction between the microflora and the host. Theoretically, there are several possible ways how this interaction takes place: (a) commensal bacteria may influence the mucosal immune system indirectly via uptake of whole bacteria or their fragments by either M cells, dendritic cells that send dendrites into the mucus layer, or via passive penetration of whole bacteria into the mucosa via incidental defects in the epithelial lining and mucus layer (e.g. due to a viral enterocolitis). (b) commensal bacteria may affect epithelial cells directly via epithelial Toll like receptors with affinity for bacterial cell wall components or via small 'soluble signals' that diffuse through the gelatinous mucus layer. Commensal bacteria that are responsible for the interaction with the host are expected to be in close vicinity with the epithelial cells and the mucosal immune system; these bacteria may be present within the mucus layer or be attached to epithelial cells. It is commonly believed that the composition of the bacterial microflora within the mucus layer differs from the microflora in the intestinal lumen. An mucosa-adherent bacterial population, if it exists, should have the capability to attach to the mucus layer or to epithelial cells and multiply within the mucus and, thus, form microcolonies. In contrast, lumenal bacteria must be capable to multiply under the strict anaerobic conditions within the lumen and be able to degrade lumenal fibers and other remnants and use these as nutrients.

Hypothesis for the pathogenesis of Crohn's disease

IBD has a multifactoral origin. In the normal intestine a number of systems exist to avoid inflammation. There must be a redundancy of mechanisms so that failure of one mechanism is not enough to cause active inflammation. Therefore we hypothesize that IBD exacerbations may be the result of a 'two hit' process: (1) a temporary (lasting months or years) general loss of mucosal tolerance for luminal antigens (in the colon: anaerobic bacteria) together with (2) a temporary deficiency of either (a) the mucosal barrier function or (b) the capacity to remove antigens out of the mucosa without causing inflammation.

Outline of this thesis

The aim of this thesis is to investigate the interaction of the commensal colon microflora with the mucosal immune system in healthy subjects and patients with IBD. We particularly focussed on the humoral immune response. As a measure of the mucosal humoral immune response towards commensal colon bacteria we analysed fecal bacteria for the presence of immunoglobulins coated onto their surface. Presumably, most of these immunoglobulins are produced in the mucosa. A considerable percentage of the bacteria are not coated with immunoglobulins. For these bacteria, the mucosal immune system may be tolerant assuming that these bacteria have had ample time to come into contact with the immune system of the host. In chapter 2 we describe a newly developed flow cytometry method to analyse the presence of immunoglobulins on fecal bacteria. In chapter 3 fecal suspensions of 22 healthy volunteers were analysed and the percentage bacteria that were coated with IgA, IgG or IgM were determined. In chapter 4 this method was applied to fecal suspensions of patients with infectious colitis and IBD with various disease activity. Finally in chapter 5 this method was extended with double staining with fluorescent rRNA targeted probes (FISH) in order to determine whether certain bacteria are preferentially coated with immunoglobulins. Commensal bacteria that are responsible for the interaction with the host are expected to be in close vicinity with the epithelial cells and the mucosal immune system. However, it is not known whether these bacteria are present within the mucus layer or attached to epithelial cells. Furthermore, it is not known whether the composition of the bacterial microflora within the mucus layer differs from the composition of the microflora in the intestinal lumen (feces). With
FISH it is possible to specifically stain bacteria in biopsy sections. In chapter 6 we describe FISH of biopsies out of 6 different regions of the colon and terminal ileum of 9 healthy subjects in comparison with FISH of their fecal flora.

Literature


34. Lu L, Walker WA. Pathologic and physiologic interactions of bacteria with the gastrointestinal epithelium. Am J Clin Nutr 2001;73(suppl):1124S-30S.


