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REVIEW

Evidence for the involvement of infectious agents in the pathogenesis of Crohn's disease

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Abstract

Many advances have been made in the understanding of Crohn's disease (CD) pathogenesis during the last decade. CD is currently seen as a predominantly T-lymphocyte-driven disease characterized by the presence of a complex cocktail of interacting cytokines, chemokines and other mediators produced by a variety of cell types. Prevailing theories of CD pathogenesis suggest that patients' T-lymphocytes are inappropriately activated in the setting of an immune imbalance, which is itself caused by an unfortunate confluence of genetic and environmental factors. The T-cell response then leads to the chronic inflammation characteristic for the disease. Various environmental factors may play a role in the development of CD, but microbes are most consistently implied. This theory is based on epidemiological, clinicopathological, genetic and experimental evidence. Despite the abundance of arguments for the implication of bacteria in the etiopathogenesis of CD, the precise role of bacteria in this disease still remains elusive. Three not necessarily mutually exclusive theories have been proposed: (1) an unidentified persistent pathogen; (2) an abnormally permeable mucosal barrier leading to excessive bacterial translocation; and (3) a breakdown in the balance between putative "protective" versus "harmful" intestinal bacteria ("dysbiosis"). At present, one cannot exclude with certainty any of these three proposed hypotheses; they may all apply to CD to a certain extent.

Key words: Crohn's disease; Etiology; Microbiology

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INTRODUCTION

Many advances have been made in the understanding of Crohn's disease (CD) pathogenesis during the last decade. CD is currently seen as a predominantly T-lymphocyte-driven disease characterized by the presence of a complex cocktail of interacting cytokines, chemokines and other mediators produced by a variety of cell types. Prevailing theories of CD pathogenesis suggest that patients' T-lymphocytes are inappropriately activated in the setting of an immune imbalance, which is itself caused by an unfortunate confluence of genetic and environmental factors. The T-cell response then leads to the chronic inflammation characteristic for the disease.

MICROBES, AN ENVIRONMENTAL FACTOR IMPLIED IN CD PATHOGENESIS

Various environmental factors such as smoking, appendectomy and the composition of the diet may play a role in the development of CD[1-6]. Microbes are however the most consistently implied factor in CD pathogenesis. This theory is based on epidemiological, clinicopathological, genetic and experimental evidence.

Epidemiological evidence

Evolutions in the epidemiology of CD during the second half of the 20th century may be explained in part by changes in the exposure of the human intestine to microbes over the same period. Some investigators propose that increased hygiene and the concomitant delayed exposure to enteric pathogens have contributed to the surging incidence of CD[7]. Not only the timing but also the type of microbes to which the gut is exposed may be important[8]. Changing dietary practices such as decreased consumption of oats, rye and bran, increased consumption of uncooked pork, unpasteurized milk and cheeses, and drinking well water instead of tap water may all favour a shift from a "protective" to a "harmful" gastrointestinal (GI) flora[9]. The "cold chain hypothesis" postulates that

CD results from an increased and chronic exposure to microbes which are able to survive at low temperatures (so-called psychrotropic bacteria, such as *Listeria monocytogenes*, *Yersinia enterocolitica*, *Clostridium botulinum* and *Bacillus cereus*). The chronic ingestion of these bacteria, new in the 20th century, would result from the increasingly common use of refrigeration in food preparation and storage, with the domestic refrigerator as the final step in the cold chain[10]. Another possible cause of sustained alterations in the gut microbiota is the increased use of antibiotics in human and veterinary medicine. Insufficient doses of antibiotics can induce a capacity for toxin production in bacteria or can make them invasive. It has been documented that prior antibiotic therapy can promote the outbreak of CD, and antibiotic use 2-5 years pre-diagnosis has an odds ratio of 1.32 in CD incident cases after adjusting for age, sex, smoking and the use of other drugs[11,12].

Clinicopathological evidence

The clinicopathological features of incident and recurrent CD, including its extra-intestinal manifestations, can be mimicked to a certain degree by specific enteric infectious diseases. Most notable in this regard are *Campylobacter*, *Salmonella*, *Shigella* and *Yersinia* infections. These can all present with an endoscopic picture of focal/segmental enterocolitis. *Yersinia* species (spp) may persist for a long time in the bowel wall and sustain chronic inflammation with its attendant consequences for tissue architecture. Differential diagnosis is by stool or blood culture or serology, as biopsies can show focal active colitis reminiscent of CD[13-22]. Other infectious microorganisms, which can cause diagnostic confusion, are *Escherichia coli (E. coli)* serotype O157:H7[23], *Chlamydia trachomatis*[24], *Mycobacterium tuberculosis*[25], *Actinomyces* spp[26], *Entamoeba histolytica*[27], *Giardia lamblia*[28], *Blastocystis hominis*, *Cryptococcus neoformans* and *Histoplasma capsulatum*.

Diarrhoea is a major symptom of CD, but also of infectious gastro-enteritis. This may occasionally cause differential-diagnostic problems. Infection with enteric viruses such as rotavirus, Norwalk-agent or adenovirus may provoke an exacerbation of pre-existing CD and thus enters in the differential diagnosis of CD relapse[29]. Acute bacterial enteritis can be confused with a first attack of CD, especially if stool cultures remain negative. Fulminant CD colitis with toxic dilatation, while being rare, is commonly treated surgically. These cases may be confused with infection by *Entamoeba histolytica*, *Salmonella* spp, *Campylobacter jejuni* and *Clostridium difficile*, which can sometimes be treated medically[30,31]. Chronic diarrhoea in immunocompetent patients is generally caused by non-infectious diseases like CD. The three most common infectious causes are *Giardia lamblia*, *Entamoeba histolytica* and *Clostridium difficile* (the latter can be provided with a niche by previous use of antibiotics). The clinicopathological manifestations in all three can be confused with CD. Clinical activity of CD may diminish with CD4 T-cell depletion in AIDS. On the other hand, very low CD4-counts may allow the development of AIDS-related GI infections[32]. Microscopic examination of a colonoscopic biopsy set may be helpful in the differential diagnosis between infectious gastro-enteritis and a first attack or relapse of CD. Infectious disease usually shows a picture of acute self-limited colitis, although this may evolve over time in focal active colitis. In such cases, differential diagnosis from CD becomes more difficult[33]. It should be noted that the typical histological features of chronic inflammatory bowel diseases (basal plasmocytosis, crypt distortion, villous colonic mucosa, crypt atrophy) increase in frequency with longer disease duration[34].

Infections may also arise as side-effects of CD therapy. Induction and maintenance of remission in active disease are usually obtained by suppressing the adaptive immune system. The attending risk is a re-activation of suppressed infectious agents or the creation of new opportunities for opportunistic pathogens. Examples of such infectious agents are *Clostridium difficile*, *Mycobacterium tuberculosis*, *Aspergillus spp*, cytomegalovirus, varicella-zoster virus and Epstein-Barr virus. The surgical treatment of CD can be complicated by abdominal wound sepsis (e.g. by *E. coli*, *Proteus spp* and *Staphylococcus aureus*), unless antibiotic prophylaxis was applied. The increased risk of surgical infections may be related to the high degree of bacterial translocation in CD[35].

It has been observed that CD recurrence in the neoterminal ileum after curative ileal resection depends on the presence of the faecal stream[36]. Histopathological and electron microscopic studies have shown that intestinal contents trigger postoperative recurrence proximal to the ileocolonic anastomosis already in the first days after surgery[37]. Metronidazole therapy for 3 mo decreases the severity of this early endoscopic recurrence and seems to delay symptomatic recurrence as well[38]. Likewise, ornidazole treatment for up to 1 year significantly reduces the endoscopic and clinical recurrence rate of CD after surgery[39]. The construction of a nipple valve may delay and perhaps prevent symptomatic recurrence[40]. A possible explanation is that the anastomotic configuration may influence colonization rates in the neoterminal ileum. When compared with controls, the neoterminal ileum in CD patients is more heavily colonized by *E. coli*, enterococci, bacteroidetes and fusobacteria[41].

Genetic evidence

In 2001, two independent groups identified the CARD15 gene on chromosome 16q as a susceptibility gene in CD. Three common single-nucleotide polymorphisms (SNPs) of this gene were found to be associated with development of CD: two missense mutations, Arg702Trp (SNP8) and Gly908Arg (SNP12), and a frameshift mutation, Leu1007fsinsC (SNP13)[42,43]. The CARD15 protein functions as an intracellular receptor for muramyl dipeptide (MDP), the minimal structural motif of peptidoglycan, a common component of gram-positive and gram-negative bacterial cell walls. MDP may enter the cytosol via the transporter protein hPepT1 and then binds to the C-terminal leucine-rich repeat (LRR) domain of the CARD15 protein[44]. Upon recognition of MDP, CARD15 activates the nuclear factor kappa B (NF-κB) pathway via a Rick/Rip2 interaction[45]. Because NF-κB is a key factor in the inflammatory response, CARD15 is considered as a pro-inflammatory molecule. The CD-associated mutations of the CARD15 gene affect the LRR domain of the protein and thus may impair its pro-inflammatory function. This

has potentially serious consequences given the normal expression of CARD15 in cells of the monocyte lineage and in Paneth cells, which are both involved in antibacterial defense[46-48]. The increased odds ratio for CD in double-dose CARD15 mutation carriers, the impaired defensin production by Paneth cells in ileal CD[49,50], data from *in vitro* models of transiently transfected cell lines[51-53] and a loss of response to MDP observed in *ex vivo* analyses of circulating monocytes from CD patients[54] all suggest a loss-of-function model for CARD15 in CD. It seems paradoxical that loss of function in a pro-inflammatory molecule such as CARD15 can cause an inflammatory condition such as CD. Most authors propose that defective recognition of bacterial components allows an increased bacterial proliferation in the gut, which in turn would be a powerful stimulus for the adaptive immune system. The end result might seem a T-lymphocyte-driven disease. Attractive as this theory is, it remains however at present wholly speculative. It should be noted that other explanations for the paradox have been offered as well[55-58].

Experimental evidence

There are currently more than 20 animal models of chronic inflammatory bowel disease. These models can be divided into 4 groups: (1) genetically induced as a result of deletion (e.g. $IL10^{-/-}$, T-cell receptor mutant mice) or overexpression of genes (e.g. IL7 transgenic mice, HLA-B27 transgenic rats); (2) adoptive transfer models (e.g. the CD45RB transfer model, the heat shock protein 60 CD8 T-cell transfer model); (3) inducible colitis models (e.g. TNBS- or DSS-induced colitis); and (4) spontaneous colitis models (e.g. C3H/HejBir mice, SAMP1/Yit mice)[59]. All the genetically engineered mouse models require commensal enteric bacteria for the development of intestinal inflammation. Various organisms including *Helicobacter* spp and *E. coli* have been implicated[60,61]. The role of the flora in the initiation of inflammation may be host-dependent. Development of inflammation in these animal models further requires a particular genetic background and the presence of T-helper cells[62].

THE PRECISE ROLE OF BACTERIA IN THE PATHOGENESIS OF CD

Despite the abundance of arguments for the implication of bacteria in the etiopathogenesis of CD, the precise role of bacteria in this disease still remains elusive. Three not necessarily mutually exclusive theories have been proposed: (1) an unidentified persistent pathogen; (2) an abnormally permeable mucosal barrier leading to excessive bacterial translocation; and (3) a breakdown in the balance between putative "protective" versus "harmful" intestinal bacteria ("dysbiosis").

The unidentified persistent pathogen theory

The similarities between CD and some forms of infectious enterocolitis are sufficiently evident that numerous specific microbial aetiologies for CD have been proposed over the years. One of the first descriptions of what later became known as "Crohn's disease"[63] was made by Dalziel in 1913[64]. He noted similarities between his cases of "chronic interstitial enteritis" in humans, and Johne's disease in cattle, which is caused by *Mycobacterium avium subsp paratuberculosis (MAP)*. He therefore suggested that *MAP* may have been the cause of the disease which he described. This hypothesis has since been tested extensively by numerous investigators, and evidence has accumulated both in favour and against the theory. *MAP* has been cultured from CD tissues[65] and peripheral blood of CD patients[66], although not uniformly[67]. The variability in the results may be partly due to the fastidious culture requirements of the cell wall-deficient forms ("L-forms") of *MAP*, which are reportedly present in CD. Detection rates for *MAP* DNA in CD tissues using a specific PCR range everywhere from 0% to 100%[68-70]. This large variation may be due to differences in DNA extraction techniques or to geographical variations in the prevalence and modes of transmission of *MAP*[71]. Investigation of the presence of an immune response to *MAP* antigens in CD patients has also yielded both positive and negative results[72,73]. Enthusiasm for *MAP* as the causative agent of CD has further diminished after the recent publication of the results of a large, prospective, placebo-controlled, double-blind, randomized trial of 2 years clarithromycin, rifabutin and clofazimine in active CD, with a further year of follow-up included in the study design[74]. Although after 16 wk treatment significantly more subjects were in remission in the antibiotic arm than in the placebo arm, this benefit could not be sustained later on. After two years, the relapse rate was similar in the antibiotic and the placebo group. The good result in the first 16 wk may have been due to concomitant administration of a tapering course of prednisolone, which can have non-specific bacterial effects. Numerous other specific bacteria have been proposed as candidate causative agents of CD, including *Pseudomonas maltophilia*[75], *Mycobacterium kansasii*[76], *Chlamydia trachomatis*[77], *Bacteroides fragilis*[78] and *Listeria monocytogenes*[79]. Like *MAP*, *P maltophilia* and *M kansasii* were first isolated from CD tissues as L-forms. This is by no means a rare phenomenon as a variety of other bacteria such as *Enterobacteriaceae*, *Staphylococcaceae* and *Streptococcaceae* have been cultured from tissues of IBD patients (but not from control subjects) as L-forms as well. Recovery rates of these L-forms are apparently not influenced by previous medical treatment. This suggests either that L-forms may be involved in disease causation, or that their presence in mucosal biopsies is a result of the disease process itself[80]. Adherent-invasive *E. coli* (AIEC) is a specific pathovar which has been isolated from the intestinal mucosa of CD patients. The reference strain LF82 is able to adhere to intestinal epithelial cells, to invade these cells via a mechanism involving actin polymerization and microtubules organization, and to survive and replicate within macrophages[81-83]. Recently, it was shown that pathogenic B2+D *E. coli* strains are significantly more abundant in IBD patients than in controls. The B2+D phylogenetic groups are associated with the presence of serine protease autotransporter proteins and adherence factors and may have a significant role in disease aetiology[84].

The excesssive bacterial translocation theory

Bacterial translocation can be defined as the passage of

viable endogenous bacteria from the GI tract to mesenteric lymph nodes and other internal organs. The proportion of surgically treated CD patients with bacterial translocation prior to antibiotics administration varies between 30% and 50%, which is much higher than in controls (5%-15%). This suggests that bacteria leak from the bowel lumen in a high proportion of CD patients. The translocating bacteria may be involved in the pathogenesis of fistulas and abscesses, and translocation may predispose patients undergoing laparotomy to the systemic inflammatory response syndrome. Bacterial strains involved in translocation include *E. coli*, *Enterococcus* spp, *Clostridium perfringens*, *Proteus* spp and *Bacteroides fragilis*. Distension of the intestine proximal to a stricture may predispose to bacterial translocation[85,86].

The dysbiosis theory

The human gut contains a large number of microorganisms, most of which belong to the domain Bacteria. The intestine of an adult human individual may contain more than 500 different bacterial species at any moment, with 30-40 species comprising up to 99% of the total population[87]. The concentration of bacteria increases along the small bowel from approximately 10^4 in the jejunum to 10^7 colony-forming units (CFUs) per g of luminal content at the ileal end, with predominance of gram-negative aerobes and some obligate anaerobes. The colon is heavily populated by strictly anaerobic bacteria at concentrations of approximately 10^{12} CFUs per *g* of luminal content[88]. The traditional method to investigate the composition of the gut flora is culture-based: bacteria are isolated on selective growth media and identified by phenotypic characteristics. However, over 50% of the bacterial cells observed by microscopic examination of a faecal specimen cannot be grown in culture. Molecular biologic techniques have recently been developed to characterize these bacteria. These new techniques are largely based on the sequence diversity of the 16S rRNA gene. This gene is essential for the survival of all bacteria, since it encodes a part of the ribosomal RNA. Its sequence contains well-preserved and more variable parts. The variability between the 16S rRNA gene sequences of two bacterial species can be used as a measure for their phylogenetic distance. Alternatively, the 16S rRNA gene sequence can be used to identify or characterize the bacteria present in a biological specimen, even if they are not cultivable. A large number of 16S rRNA gene sequences (> 79 000) are currently available in public databases[89].

Several studies using different methods have repeatedly shown that the faecal microflora differs between CD patients and healthy controls, although the reported changes are not always consistent[90]. Bowel contents in quiescent CD are characterized by high total viable counts, high biodiversity and temporal stability. There is an increased number of obligate anaerobic Gram-negative rods, e.g. *Bacteroides fragilis* and *B vulgatus*. Many unusual species are also present in low numbers. On the other hand, counts of butyrate-producing Firmicutes (especially those of the *Clostridium leptum* group) are typically diminished. This may lead to an increased frailty of the intestinal epithelium[91-97]. The observed changes are independent of disease location, the presence of anatomical abnormalities secondary to inflammation or scarring, treatment with sulphasalazine or corticosteroids and even surgery. It has therefore been suggested that the faecal flora abnormalities in CD may be due to constitutional factors[98-102]. CD relapse causes huge disturbances in faecal flora composition. With diarrhea, total viable counts diminish due to a decrease in the number of strictly anaerobic bacteria (e.g., bifidobacteria). There is also a shift towards facultative anaerobes (particularly *E. coli*)[103,104].

The alterations in the luminal or faecal flora in CD patients are however not necessarily relevant for what happens at the mucosal level. Most studies of the mucosal flora report an increased likelihood to obtain positive culture results and higher total viable counts in CD patients' biopsies than in controls, especially in the ileum. Mucosa-associated bacteria in CD typically form a thick, densely populated biofilm associated with the mucus which covers the intestinal epithelium. Bacterial concentrations are higher in normal-looking CD mucosa than in biopsies obtained from visibly inflamed areas[105-111]. Not only the abundance but also the composition of the mucosa-associated flora is abnormal in CD. In general, the prevalence of the phyla Proteobacteria and Bacteroidetes is increased at the expense of the phylum Firmicutes[112]. Facultatively anaerobic bacteria (enterobacteria and enterococci) are found more frequently in ileum biopsies from CD patients than in normal subjects[113]. Taken together, the literature data suggest that the mucosal flora in CD patients is abnormal even before the onset of inflammation[114].

CLINICAL IMPLICATIONS

Given the abundant evidence for a role of bacteria in the pathogenesis of Crohn's disease, it has been suggested that the intestinal bacterial flora can be manipulated for therapeutic purposes. Several investigators have reported on the use of antibiotics and probiotics in CD patients. From these data, it appears that treatments must be individualized, depending on disease location and activity[115].

Antibiotics

Antibiotic therapy in Crohn's disease may decrease the number of mucosa-associated bacteria, kill or suppress bacterial variants which induce early histological lesions, and reduce the rate of bacterial translocation. Broad-spectrum antibiotics are frequently and successfully used in the treatment of mild to moderate CD, although no large controlled trials have yet been performed[116].

Metronidazole has been most extensively investigated. This antibiotic is active against strictly anaerobic bacteria. It has been used in monotherapy, especially with colonic and ileocolonic CD, but not for ileitis[117-120]. It has however many side-effects such as nausea, anorexia, dysgeusia, dyspepsia and peripheral neuropathy, which may limit its use.

Other antibiotics have also been tested. Ciprofloxacin therapy is one of the potential treatments of active CD, and is also effective when used in combination with standard treatment in patients with resistant disease[121,122]. Combined ciprofloxacin plus metronidazole treatment has

been shown to be a potential alternative to steroid therapy in the acute phase of CD, although it is more effective in colonic than in isolated small bowel disease[123,124]. The combination of ciprofloxacin and metronidazole is also used in the treatment of perianal CD[125]. Other antibiotics which have been tested are rifaximin[126] and clarithromycin[127].

Probiotics

Probiotic therapy is one possible approach to the prevention of relapse in CD patients. Several microorganisms such as *E. coli* Nissle, *Lactobacillus GG* and *Saccharomyces boulardii* have been tested. At present however, there is no evidence that these probiotic preparations perform better than placebo or aminosalicylates in this setting. The use of probiotics as a maintenance therapy for medically or surgically induced remission in CD is therefore currently not recommended[128].

CONCLUSION

At present, one cannot exclude with certainty any of the three proposed hypotheses on the role of bacteria in CD etiopathogenesis. Uninflamed bowel mucosa in CD may be heavily colonized by facultative anaerobe and/or aerotolerant bacteria, possibly due to a genetically determined defect in the capacity of the mucosa to "hold back" such bacteria. The ecological conditions prevailing in this mucosa-associated biofilm may then enable some bacteria to acquire particular virulence traits by horizontal gene transfer. Eventually, pathovars may arise which have an increased capacity for adherence to and invasion of the mucosa. Aphtoid ulcerations may develop in this setting. Once the mucosal integrity is broken down, bacterial translocation through the bowel wall is greatly enhanced and may lead to the development of complications such as fistula and abscesses. The human body may then react with a basically appropriate but uncontrollable inflammatory response which leads to further tissue damage. While this disease causation theory stresses global disturbances in the composition of the bowel flora and increased bacterial translocation, it cannot be excluded that specific bacteria may be particularly damaging to people who are genetically susceptible to the development of CD. The adherent-invasive *E. coli* pathovar is perhaps the most likely candidate in this regard.

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