The Role of Microbes in Crohn’s Disease

Paul B. Eckburg¹,²,³ and David A. Relman¹,²,³

¹Department of Microbiology & Immunology and ²Division of Infectious Diseases & Geographic Medicine, Department of Medicine, Stanford University School of Medicine, Stanford, and ³Veterans Affairs Palo Alto Health Care System, Palo Alto, California

Despite decades of research, the etiology of Crohn’s disease (CD) remains unknown. Its pathogenesis may involve a complex interplay between host genetics, immune dysfunction, and microbial or environmental factors. Microorganisms, including pathogens and members of the indigenous microbiota, may initiate or propagate the inflammatory process in CD. The pathogenesis of CD has been difficult to study, owing to the broad spectrum of typically nonspecific clinical manifestations, the complexity of environmental and genetic factors, the lack of an accurate model of disease, and the limitations of microbiological methods. A more useful and relevant paradigm for the etiology of CD might be based on the idea of a pathogenic microbial community profile and might emphasize the role of interactive sets of microbes, rather than the role of individual organisms. We review how microbes may participate in the pathogenesis of CD and how they may inappropriately activate the mucosal immune system in genetically predisposed individuals.

IS CROHN’S DISEASE (CD) AN INFECTIOUS DISEASE?

Upregulated immune responsiveness to the intestinal microbiota or specific members thereof may be the central event in the pathogenesis of inflammatory bowel disease (IBD), whether classified as CD or ulcerative colitis (UC). However, UC differs from CD in its clinical manifestations and hypothesized pathogenic mechanisms. Inflammation in UC typically involves the colon in a continuous distribution, whereas CD usually involves the ileum and other regions of the alimentary tract in a patchy, segmented manner [1]. Unlike CD, UC appears to be highly associated with autoimmune phenomena, including the presence of antihuman tropomyosin and antineutrophil cytoplasmic antibodies [2]. Smoking seems to protect against UC, whereas it increases the risk of CD [3]. CD displays features that more strongly suggest genetic predisposition, in that it is associated with higher rates of inheritance and twin concordance than UC [4]. Finally, CD features distinct immunopathogenic findings, including transmural gut inflammation and granulomas [5]. Although microbes may be involved in the pathogenesis of UC, this review will focus on the role of microbes in the pathogenesis of CD.

Several lines of evidence implicate microorganisms or the intestinal microbiota in the pathogenesis of CD. Some patients with CD experience improvement in clinical disease when they receive prolonged courses of antibiotics, such as oral ciprofloxacin [6] and the oral, nonabsorbable rifamycin derivative, rifaximin [7]. In addition, exposure of a surgically excluded ileum to luminal contents results in local inflammation, and diversion of the fecal stream away from the inflamed bowel is associated with disease improvement [8, 9]. IBD does not occur in germ-free animals, in contrast with their naturally colonized littermates [10–12]. Clusters of cases of CD in time and space suggest an infectious etiology [13], as does the incomplete concordance of CD among monozygotic twins [14, 15]. Finally, granulomas within the bowel wall are evident in 30%–60% of resected CD tissues [5], which may indicate an immune reaction to microbes or microbial products.

Despite a century-long search for a specific microbe as the cause of CD, no infectious agent has been linked decisively to the etiology of this disease. Difficulties exist in establishing associations between IBD and specific microbes. The exposure to potential pathogens in the gut may be transient and may occur prior to the onset of clinical disease. Microbial contact or invasion may be confined to parts of the alimentary tract that are relatively inaccessible to tissue sampling, such as the ileum or jejunum. In addition, the inciting agent(s) may be unculturable or undetectable using standard microbiological techniques. Over the past decade, applications of molecular science and technol-
ogy have illuminated the enormous complexity of the intestinal microbiota; this diversity further complicates the search for a specific infectious etiology of CD.

**ASSOCIATIONS OF SPECIFIC MICROBES WITH CD**

A number of microorganisms have been postulated to play a role in the pathogenesis of CD (table 1), but none convincingly. *Mycobacterium avium* subspecies *paratuberculosis* (Map) has been most intensely investigated. *Mycobacterium* species have been proposed to be inciting agents of CD since granulomatous ileitis and its recognized similarities to intestinal tuberculosis were originally described in the early 1900s [35, 36]. During this same period, *Map* was recognized as the etiological agent of Johne’s disease, a severe granulomatous enteritis occurring in ruminants [37]. Its histopathological similarities to human CD led to focused attempts at culturing *Map* from affected tissues. Despite the rare isolation of slow-growing mycobacteria from CD-affected intestinal tissue and mesenteric lymph node samples [16, 38], it was not until the 1980s when strains of atypical mycobacteria cultured from CD-affected tissue samples were identified as *Map* using DNA fingerprinting and hybridization techniques [18, 21]. Multiple research groups have since attempted to culture *Map* from CD-affected tissue samples or to identify serological antibody responses to *Map*-associated antigens, with inconsistent and conflicting results [38]. Various slow- and rapid-growing mycobacterial species have been isolated from IBD-affected and control tissue samples, in varying frequencies. In addition, numerous molecular studies have evaluated the presence of amplifiable *Map* DNA in tissue samples from patients who have CD, with contradictory results [20]. Indeed, across all PCR studies (most often targeting the IS900 insertion sequence of *Map*), the range of 0%–100% *Map* DNA positivity in affected tissue samples casts doubt on the role of *Map* in the etiology of CD. The fact that *Map* and its DNA have been repeatedly detected in healthy control tissue samples also casts doubt on the significance of this association with CD.

Atypical mycobacteria are ubiquitous in the environment, and *Map* has been isolated from tap water and cow milk [39]. Such data may explain the inconsistent clinical responses observed in various placebo-controlled trials of antimycobacterial drug combinations [40], in addition to the suboptimal in vitro activity against *Map* of commonly used antimycobacterial agents [41]. Discrepancies among molecular and therapeutic studies may result from differences in patient populations, severity of disease, differing experimental protocols, and unknown optimal drug regimens. Despite the paucity of supportive data, however, *Map* cannot be completely excluded as playing a role in the pathogenesis of CD.

Among nonmycobacterial microbes, several groups warrant further consideration on the basis of prior research. Epithelium-associated and invasive strains of *Escherichia coli* have been detected in tissue samples obtained from individuals with CD [24, 42]; these strains demonstrate a propensity to adhere to intestinal cell monolayers and to synthesize α-hemolysin [23]. Adherent-invasive strains of *E. coli* have been associated specifically with the ileal mucosa of patients with CD [22]. In addition, DNA sequences that are homologous to *Stenotrophomonas* (*Pseudomonas*) *maltophilia* have been associated with inflamed tissue samples from patients with CD [25], and *Pseudomonas fluorescens* has been identified as the source of a CD-associated DNA fragment called I2, which encodes for a T cell superantigen [26].

In a preliminary study, using broad-range bacterial 16S rDNA PCR, we have identified significantly more *Proteobacteria* phylum–associated 16S rDNA sequences (primarily *E. coli* and *Pseudomonas* species phylotypes) from colonic tissue samples obtained from patients with CD than from tissue samples obtained from patients with UC and from control subject tissue samples (authors’ unpublished data) [43]. Broad-range bacterial 16S rDNA PCR allows for the amplification and identification of most bacterial phylotypes in a complex microbial community, but it is limited by PCR bias [44] and by its inability to differentiate among bacteria at the strain level [45]. Whether the presence of such *Proteobacteria* phylum members in CD-affected tissue reflects a preferred colonization of inflamed tissue, the caus-

<table>
<thead>
<tr>
<th>Organism</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Mycobacterium avium</em> subspecies <em>paratuberculosis</em></td>
<td>[16–21]</td>
</tr>
<tr>
<td>Adherent-invasive <em>Escherichia coli</em></td>
<td>[22–24]</td>
</tr>
<tr>
<td><em>Pseudomonas</em> species</td>
<td>[25, 26]</td>
</tr>
<tr>
<td><em>Listeria monocytogenes</em></td>
<td>[27]</td>
</tr>
<tr>
<td><em>Mycoplasma</em> species</td>
<td>[28]</td>
</tr>
<tr>
<td><em>Chlamydia</em> species</td>
<td>[28, 29]</td>
</tr>
<tr>
<td><em>Coxiella</em> species</td>
<td>[28]</td>
</tr>
<tr>
<td>Streptococci</td>
<td>[27]</td>
</tr>
<tr>
<td><em>Helicobacter pylori</em></td>
<td>[30]</td>
</tr>
<tr>
<td>Yersinia pseudotuberculosis</td>
<td>[31]</td>
</tr>
<tr>
<td><em>Saccharomyces cerevisiae</em></td>
<td>[32]</td>
</tr>
<tr>
<td>Measles virus</td>
<td>[30, 33, 34]</td>
</tr>
</tbody>
</table>

**NOTE.** No definitive evidence or consensus expert opinion exists for any microorganism as the cause of CD.
ative organisms of inflammation, or surrogate markers of an altered microbiota remains to be resolved. More comprehensive molecular studies are needed, with larger numbers of subjects from different geographic regions, that control for various host factors and stage of disease and implement standardized tissue preparation, cultivation techniques, and molecular protocols.

THE ROLE OF THE INTESTINAL MICROBIOTA IN CD

It is not surprising that the indigenous intestinal microbiota have been postulated to play a role in the etiology of IBD, given its influence on epithelial differentiation and gut-associated lymphoid tissue assembly [46]. Recent molecular surveys have revealed the incredible diversity of this microbial community [47], whose population density in the colon may exceed trillions of organisms per gram. It is now recognized that fecal microbial populations differ significantly from those associated with the intestinal mucosa, and that each individual harbors a unique microbiota pattern [47, 48]. Multiple variables are thought to impact the composition of intestinal microbiota, including an individual’s geographic location [49], diet [50, 51], illness [52], antibiotic use [53], emotional stress [54], age [55], and host genotype [56].

Molecular methods, such as PCR and 16S rDNA sequence analysis, offer greater sensitivity than cultivation techniques in detecting the presence of microbes in complex samples. These methods have been used in small studies to assess the composition of the intestinal microbiota of patients with CD and UC and healthy subjects. Some consistent trends in the general characteristics of a CD-associated microbiota have emerged. In contrast to healthy control subjects, colonic tissue biopsy samples obtained from patients with CD tend to reveal a higher concentration of bacteria [57] and a thicker mucosal layer of bacteria [57, 58] associated with the intestinal surface; this layer of bacteria is often associated with a normal-appearing epithelium. In addition, a diverse collection of phylogenetic groups of bacteria have been detected in patients with UC and in healthy control subjects, whereas a more limited number of organisms is associated with CD. This less-diverse ecosystem consists primarily of members of the Proteobacteria and Bacteroidetes phyla [43, 58–60], in contrast to the diverse collection of Firmicutes phylum members (low guanine-cytosine content gram-positive bacteria) observed in control subjects who do not have CD. DNA digestion band pattern analysis techniques have demonstrated different community patterns between patients with active and quiescent CD and control subjects [60]; however, it is unclear whether this variation simply reflects interindividual differences among intestinal microbiota.

There are multiple problems with the design and interpretation of studies that seek a possible microbial etiology in CD. Feces has often been used as a representative specimen type for the study of the mucosal or epithelium-associated microbiota; however, the fecal microbiota differs significantly from the mucosa-associated microbiota [47, 48], and it is unclear which microbial populations or habitats may be most important in the etiology or progression of IBD. DNA digestion banding pattern analysis has been used to compare bacterial communities among relatively large numbers of specimens in an efficient manner, but this approach has relatively low resolution in discriminating among different microbes, compared with primary 16S rDNA sequence analysis [61]. No robust ecological statistical methods (e.g., as detailed in [47]) have been used to analyze large clone library sequence datasets obtained from patients with IBD, nor has a consistent “species” or phylotype definition been used in characterizing phylotypes within communities, thereby making comparisons between studies difficult. Finally, past studies have lacked consistent and sufficiently numerous controls for disease activity or severity, genetic predisposition, medications, and other host factors.

ABNORMALITIES IN THE GUT EPITHELIUM

A simple epithelial layer serves as the interface between gut microbes and host. Epithelial permeability is influenced by the integrity of the epithelial cell layer and the basement membrane, as well as by the surface mucus layer, by the autonomic nervous system function, and by the secretion of host protective factors, such as defensins. Increased epithelial permeability in IBD is postulated to play a role in facilitating mucosal penetration of antigens, which may originate in the indigenous microbiota, thereby triggering an aberrant immune response. Indeed, certain bacterial strains tend to alter bowel permeability in animal models without producing obvious physical damage to the gut epithelium (e.g., E. coli increases permeability, whereas lactobacilli decrease permeability) [62]. When the intestinal epithelial barrier is defective—whether because of experimentally altered tight junctions [63] or ablated enteric glial cells [64]—mouse models tend to be predisposed to severe gut inflammation.

Altered intestinal permeability has been documented among patients with CD, as well as among healthy relatives of patients with CD [65]. An overproduction of IgG directed against commensals occurs in patients with CD [66], potentially reflecting a breakdown in mucosal barrier function or a state of hyperresponsiveness to normally harmless bacteria. Several serologic markers have been reported to be surrogates for an abnormal immune response in CD, including antibodies directed against the Saccharomyces cerevisiae cell wall, the E. coli outer membrane porin type C, the Pseudomonas fluorescens I2 product, and the enteric bacterial flagellin CBIr1 (which is related to flagellins found in commensals of the Firmicutes phylum, such as members of the class Clostridia) [67]. Whether these antibodies reflect a result of nonspecific bacterial translo-
tion through a damaged, leaky bowel wall or a primary pathogenic process has yet to be determined. For example, it is intriguing that the transfer of CBir1-specific T cells into immunodeficient mice is sufficient to induce colitis [67]. Nonetheless, patients with CD who have elevated antibody titers directed against antigens such as E. coli outer membrane porin type C and P. fluorescens I2 are more likely to have progression to perforating or fibrostenotic disease, thereby requiring more surgery [68]. Intestinal permeability in humans with CD may also be influenced by other factors, including smoking or anti-inflammatory drug use [69]. In addition, mutations in the organic cation (OCTN) and DLG5 genes, whose products are involved in epithelial cell polarity, have been described in CD, potentially impacting epithelial permeability [70, 71].

**IMMUNE DYSFUNCTION IN CD**

The innate immune response involves the recognition of microbe-associated molecular patterns (MAMPs) by pattern recognition receptors, such as toll-like receptors (TLRs), and the subsequent elaboration of proinflammatory cytokines and chemokines. TLR expression appears to be carefully regulated to mute a proinflammatory response toward mutualistic organisms in healthy individuals. Some degree of TLR signaling may be necessary for normal mucosal barrier function and homeostasis. TLR-associated responses to MAMPs include antibacterial peptide expression, epithelial barrier fortification, and epithelial proliferation [72, 73]. However, colonic epithelial cells express relatively low levels of cell-surface TLRs, which may result in their relative “hypo-responsiveness” to bacterial lipopolysaccharides. Prolonged exposure to microbial antigens leads to a further decrease in TLR surface expression [74]. Polymorphisms in TLRs have been linked to CD [75, 76], and immunofluorescence studies have revealed that epithelial TLR expression is markedly upregulated in both UC and CD [77]. Polymorphisms in the nucleotide-binding oligomerization domain (NOD)—containing protein NOD2 (also called caspase recruitment domain [CARD]—containing protein CARD15) also play a role in CD susceptibility.

NOD2/CARD15 is a member of a group of cytoplasmic pattern recognition receptors that is expressed in many different cell types. The NF-κB pathway is induced when NOD2/CARD15 recognizes muramyl dipeptide (derived from bacterial peptidoglycan) [78]. Intestinal epithelial expression of NOD2/CARD15 appears to serve a protective function [79, 80]. The association between NOD2/CARD15 gene polymorphisms and CD susceptibility has been well documented and demonstrated in multiple ethnic groups [81, 82]. Polymorphisms have been associated with ileal involvement, young age at onset of CD, and fibrostenosing disease [83]. NOD2/CARD15 mutations, in combination with mutations at other loci (such as OCTN), may greatly increase susceptibility to CD [84]; however, additional studies are needed to determine the frequency and role of coexisting mutations such as these.

A biased Th1-type immune response also plays a role in the pathogenesis of CD [1]. In CD, mucosal dendritic cells and macrophages overproduce cytokines, leading to Th1 differentiation and inflammation within the intestinal mucosa. Genetic polymorphisms in the TNF-α and IL-10 genes have been linked to CD, which may lead to the overproduction of Th1-associated cytokines in some patients. Interestingly, the Th1-biased response in CD has been postulated to be inversely associated with the likelihood of infection by intestinal helminths, reflecting the known propensity of helminths to produce a Th2 bias [74]. The most effective treatments for CD are directed against this Th1-biased response, and include infliximab, 6-mercaptopurine, azathioprine, and anti-IL-12 antibodies [1]. Infliximab is an anti-TNF-α antibody that induces apoptosis of the T cells that produce this Th1 cytokine. 6-Mercaptopurine and azathioprine are metabolized to 6-thioguanine nucleotides, which also induce apoptosis of T cells in the intestinal lamina propria. Anti-IL-12 antibodies inhibit the action of IL-12, a cytokine produced by dendritic cells and macrophages of the lamina propria that is required for the expression of IFN-γ, which in turn plays a role in the Th1 bias in the mucosa of patients with CD.

**FUTURE DIRECTIONS**

From a clinical perspective, IBD represents a spectrum of disease that includes CD and UC and variations inbetween (e.g., “indeterminate colitis”). Given the absence of strict criteria or pathognomonic features, CD and UC are diagnosed using combinations of clinical, endoscopic, and histopathological data. These different disease phenotypes may reflect different pathogenic mechanisms and host factors, including age, prior immune experience, the presence of specific microorganisms within the microbiota, geographic location, medications, smoking, diet, and genetic predisposition. Simple questions remain unanswered: Do particular genetic polymorphisms, alone or in different combinations, predispose patients to a particular disease manifestation? Do members of the indigenous microbiota act as “pathogens,” but only in a host with a specific combination of risk factors and genetic predisposition? Or, is an abnormal microbiota pattern alone sufficient to cause CD? Does a single microorganism, such as Map, cause CD in a specific subset of patients, or could it, in turn, predispose the host to the development of a pathogenic microbiota? These and related questions need to be answered in the context of well-controlled, population-based cohort studies with large numbers of subjects and a combination of methods.

Because the gastrointestinal tract is an open system, we face a challenge in distinguishing between transient members of the microbiota and those that are more...
permanent and/or identifying well-adapted members with more-intimate host interactions. We must distinguish between cause and effect—that is, whether a particular microbial community contributes to the underlying pathology in CD or is simply selected by it. In considering the microbiota as a community “pathogen” and in building a case for causation, one might look for specific patterns of community diversity that are preferentially associated with subjects and tissues affected by CD (and not with other forms of IB and with healthy subjects), as well as microbiota composition that is predictive of subsequent CD activity and dynamic over time in a stereotypic fashion with respect to disease severity, all in the context of a susceptible genetic background. Finally, because each individual may harbor a distinct microbiota, comparisons of microbiota from IB and control subjects will be difficult to interpret without the use of appropriate inter- and intrasubject controls.

A multidimensional, interdisciplinary approach will be required to scrutinize the multifactorial pathogenesis of IB. Investigators have started to recognize the importance of creating interfaces between the disciplines of microbiology, gastroenterology, ecology, population genetics, and statistics in examining the human indigenous microbiota [47]. High-throughput molecular techniques and well-characterized samples from carefully selected patients with IB and from control subjects will be critical. Case patients and control subjects should be classified on the basis of severity of disease (measured in a variety of ways), anatomical and extraintestinal involvement, surgical history, previous and current therapy, smoking history, and genetic predisposition. Potential microbial etiologies of IB could be better evaluated in trials involving special populations of patients, such as new-onset, untreated, pediatric, or familial forms of CD. We will have to rethink the traditional approaches for proof of causation, given the complex nature of IB and the use of molecular data [85]. In addition, investigations of microbial communities could be strengthened by integrating host response profile analyses from the same tissue specimens, using such tools as microarrays [86–88], and by exploiting metagenomic approaches [59]. Continued progress in the study of IB pathogenesis will yield more precise diagnostic, prognostic, and therapeutic tools, and will reveal microbial and immune factors that are important in maintaining health.

Acknowledgments

We would like to thank Dr. Dan DiGiulio at Stanford University for his thoughtful comments on the manuscript. We would also like to thank Elies Bik, Elizabeth Purdom, Charles Bernstein, and Michael Sargent for their involvement in the preliminary analyses cited in this article.

Financial support. National Institutes of Health (AI51259).

Potential conflicts of interest. All authors: no conflicts.

References


