The Path to Crohn's Disease: Is Mucosal Pathology a Secondary Event?

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Abstract: Current models of Crohn's disease (CD) invoke an initial disturbance of the epithelial interface between the gut mucosa and intestinal microbiota. This "outside-in" paradigm, mirroring the pathophysiology of acute gastroenteritis, suggests that mucosal damage by luminal bacteria is an early, initiating factor in the etiopathogenesis of disease. However, a number of features of CD argue against a primary mucosal process, including phenotypic studies of CD patients that point to a macrophage defect and genetic studies that predict impaired innate immunity to intracellular bacteria. Intracellular pathogens, such as *Listeria, Salmonella*, and *Mycobacteria,* invade via the gastrointestinal tract with minimal or no acute mucosal pathology. These organisms then infect and persist in lymphatic tissues before inducing pathology, in the gut or elsewhere, as a secondary process. In a disease resulting from impaired macrophage responses to intracellular pathogens, mucosal damage could instead represent a terminal event in the pathogenesis of disease. Such an "inside-out" model is also compatible with observations on postoperative disease relapses where subepithelial pathology precedes ulceration. This alternative disease paradigm suggests that clinical and experimental research efforts should be directed at deeper processes in the gut wall and attached mesentery to understand how intracellular bacteria could initiate or exacerbate this chronic inflammatory disease.

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Key Words: Crohn's disease, pathogenesis, mucosal damage

The application of genetic, and more recently genomic, methodologies to the study of Crohn's disease (CD) has resulted in a series of spectacular successes.[1–5] The finding of specific genes, and their associated pathways, that are implicated in the pathogenesis of this disease now poses a new challenge for the understanding of this enigmatic disease. As a general rule, new genomic data provide little insight into disease processes when considered in isolation; therefore, a thorough working knowledge of the disease process is required to best interpret the findings. However, the greatest conceptual benefit of genome-wide studies is the absence of a preconceived hypothesis, permitting the data to suggest new perspectives on old problems, potentially refuting dogma along the way.[6] In the case of CD, it is therefore pertinent to ask whether one can simply overlay new genomic results onto traditional models of disease, or rather, whether an entirely new paradigm of disease pathogenesis might be indicated.

While clinical diseases are typically considered "state functions," where the route taken to disease is immaterial, disease models represent "path functions," where the path taken from a state of health to a state of disease is critical to understanding the specific molecular and cellular events. The etiologic pathway to disease is often unnecessary when treating an individual patient, but nonetheless a familiar concept to most physicians who have been asked by their patient: "Why did I get sick?" The etiologic pathway to disease, however, is necessary for the design of rational therapeutic or preventive strategies, where one aims to uncover the earliest steps where interventions may be beneficial. For this reason, disease models deliberately include arrows and impose directionality to predict the sequence of events that ultimately results in the disease state. Importantly, not all patients sharing the same disease state have forcibly traveled the same path. For instance, pulmonary tuberculosis (TB) can follow inhalation of *Mycobacterium tuberculosis* from a contagious case or ingestion of *Mycobacterium bovis* from a contaminated dairy product. Both scenarios result in the state of TB. The differing routes of exposure both explain how patients get sick and guide preventive strategies, whether it involve respiratory isolation of TB cases or pasteurization of milk.

In the case of CD, decades of clinical-pathologic correlation have generated a set of criteria that define the disease state, allowing for the possibility of some phenotypic heterogeneity between disease subsets.[7] However, the path to this clinical diagnosis remains largely unknown. Previously classified as an autoimmune disease, CD is increasingly being viewed as the result of an impaired or dysregulated host response to intestinal microbiota.[8–10]

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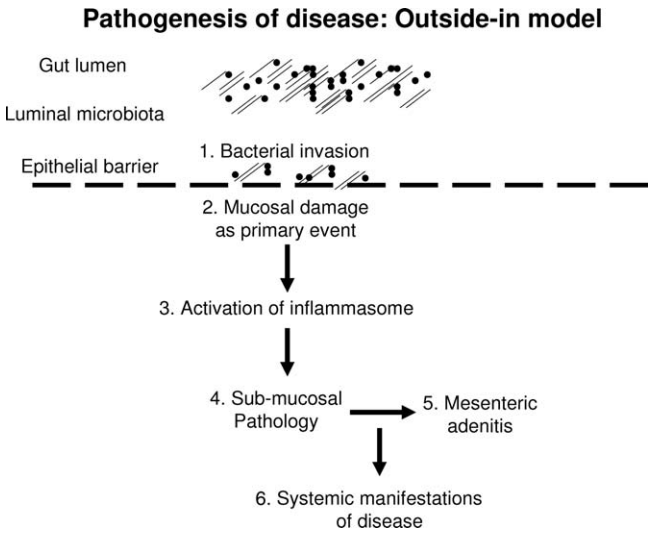

FIGURE 1. The conventional model of CD pathogenesis invokes a primary mucosal insult as an initiating event and emphasizes the centrality of inflammasome activation in the disease. In this model, submucosal pathology occurs as a consequence of a primary event at the mucosa and mesenteric adenitis is a secondary phenomenon, clinically termed 'reactive lymph nodes.'

This paradigm shift was anticipated by clinical investigation of the immune response of CD patients[11,12] and the observation that patients with chronic granulomatous disease share many of the pathologic features of CD in the gut.[13] Indeed, these observations argue toward an immune deficiency, as opposed to an overactive immune response, as critical to the development of CD.[14,15] This possibility has received further support from genetic and genomic studies that have converged on genes involved in the innate immune response to bacterial infection.[16] Therefore, a most contemporary working model invokes an immunologic defect plus the presence of certain bacterial exposures, stimulating a variety of experimental models that aim to dissect the mucosal immune response to intestinal microbiota as a function of defined chemical, genetic, or immunologic perturbations.

While the additional insights gained from genomic studies have solidified the importance of bacterial triggers in the pathogenesis of CD, it is not clear that the path from a genetically susceptible host to a patient with disease follows the most direct route, i.e., from the lumen into the bowel wall. In this essay I argue that contemporary models of CD suffer from an unproven directionality that may hinder both conceptual approaches to understanding this disease and experimental models aimed at dissecting critical processes in disease pathogenesis. Specifically, an "outside-in" model of disease, analogous to the process of acute bacterial gastroenteritis (Fig. 1), directs investigators to the interface between intestinal microbiota and the host, relegating deeper processes to secondary or reactive events. An alternative "inside-out" model of CD (Fig. 2) addresses a number of deficiencies with the former model and focuses attention on the depth of this transmural and systemic disease. While experimental systems based on both models can successfully provoke a state of bowel inflammation, the proximal events are so markedly different that different sets of investigation are needed on different time scales to adequately dissect the respective paths to disease. By making unsubstantiated assumptions about the path to disease, I propose that investigators risk overlooking other possible routes, from a bacterial trigger in an immunocompromised host to a chronic inflammatory disease, routes that might be amenable to experimental dissection, and ultimately, intervention.

CD AS AN "OUTSIDE-IN" DISEASE

The intestine is known to harbor an impressive collection and concentration of bacteria. Therefore, it follows

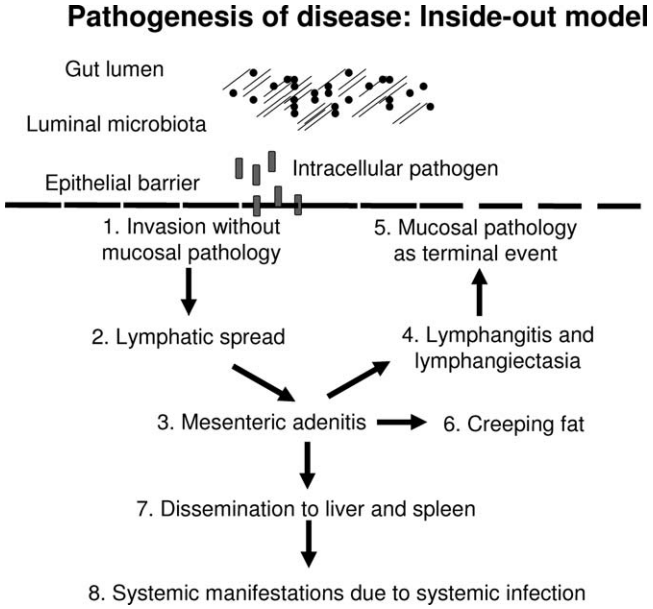

FIGURE 2. The alternative model of CD pathogenesis proposes that infection by intracellular bacteria and invasion to regional lymph nodes occurs without frank mucosal pathology, in both resistant and susceptible hosts (steps 1–3). In this model, effective immune responses in resistant hosts lead to clearance of pathogens from the lymph nodes, with resolution of lymphadenitis. In the susceptible host, persistent infection in the mesenteric lymph node(s) is central to the disease process and eventually leads to mucosal disease (steps 4–5, via lymphatic obstruction), mesenteric disease (step 6, via proximal spill-over of infection), and distal manifestations of disease (steps 7–8, via systemic spread of infection). Here, mucosal ulceration is hypothesized to be a terminal event, which may paradoxically be the first sign of disease by endoscopy.

that these organisms must be appropriately contained so as to prevent locally invasive and disseminated infections. Because many of these organisms produce proinflammatory molecules, such as lipopolysaccharide (LPS), it is tempting to propose that the overabundance of certain organisms, and/or their metabolic products, may provoke an inflammatory state in the host. Conversely, it should also be noted that the normal outcome between the human gut and the bacterial flora is a state of health. Therefore, the presence of bacteria in the gut lumen, on its own, is insufficient to trigger inflammatory bowel disease (IBD).

In contrast to the normal détente between the human host and intestinal microbiota, the introduction of specific pathogenic organisms results in gastroenteritis. Among these, enteropathogenic strains of *E. coli* (EPEC) have been convincingly shown to cause human disease via human volunteer studies, serving as the basis for mechanistic studies that have dissected the specific steps in infection, invasion, and induction of pathology.[17] From these studies it is known that EPEC, unlike commensal strains of *E. coli*, harbor genes coding for virulence factors that enable bacterial attachment to the apical surface of the enterocyte and injection of bacterial effectors into the host cell.[18] The result of this nonphysiologic interaction between host cells and pathogenic bacteria is cell death, inflammation, and clinical disease.[19] A key question is whether this type of "outside-in" process is also implicated in the etiology of CD.

While previously CD was often approached as an autoimmune disease, the potential role of intestinal microbes was already predicted a few decades ago, for instance, by Strober,[20] who wrote in 1985: "… no matter what the initial immunological disorder may be, the mechanism underlying the gastrointestinal inflammation ultimately comes to involve a response to materials in the mucosal environment." This notion was developed a decade later by Sartor[21]: "Nonspecific intestinal inflammation can be induced by a wide variety of enteric infections or ingested toxins. The vast majority of hosts respond to these injurious events by promptly down-regulating the inflammatory response …. The genetically susceptible host, however, who lacks the ability to suppress the inflammatory response efficiently, has inappropriate amplification of the immune cascade." Despite the impressive advances from genomic study of this disease, more recent reviews still focus on the same site. For instance, Xavier and Rioux wrote in 2008: "The mucus layer and tight junctions associated with intestinal epithelial cells maintain barrier integrity under homeostatic conditions. Disruption of this dynamic balance between host-defence immune responses and luminal enteric bacteria at the mucosal frontier is central to the pathogenesis of Crohn's disease."[22] The greatest potential benefit of a genome-wide study, namely, revisiting this disease with a fresh and novel perspective, has yet to be fully realized.

PROBLEMS WITH CD AS AN "OUTSIDE-IN" DISEASE

Three key issues with the "outside-in" paradigm are: 1) "normal flora," 2) predictions from genetic studies, and 3) clinical investigations that point to a macrophage defect.

The definition of normal flora is a relatively nebulous concept pending completion of the human microbiome project. However, accepting that we do not know the composition of intestinal flora in great detail, we can operationally define normal flora as a statistical concept. Since the highest reported incidence of CD is about 20 per 100,000,[23] it follows that 99.98% of people do not develop CD each year. Moreover, even in those at highest risk, such as subjects homozygous for predisposing *NOD2* mutations, over 95% do not develop disease.[24] Statistically speaking, disease is not the normal outcome of the host interacting with his or her intestinal microbiota, even in the face of host polymorphisms that increase the risk of CD by 30–40-fold.[25] Consistent with this, long-term observations of *Nod2−/−* mice interacting with their intestinal flora failed to detect chronic enteritis.[26,27] Normal flora is not sufficient to cause disease. Invoking gut flora as the cause of CD is analogous to invoking skin flora as the cause of psoriasis.

Genetic studies have used either linkage analysis or genome-wide association methods to ask which loci, and subsequently which genes, are nonrandomly associated with the occurrence of disease. To date, a large number of genes have been reported in studies of IBD, but only 3 are specifically linked to CD: *NOD2, ATG16L1,* and *IRGM*.[28] The protein NOD2 is located in the cytosol, where it recognizes intracellular bacterial peptidoglycan, namely, muramyl dipeptide (MDP).[29,30] The other CD susceptibility genes, *ATG16L1* and *IRGM*, code for proteins implicated in autophagy, a process involved in the elimination of intracellular pathogens.[16,31–34] Together, these findings argue that the greatest import of mutations in CD-associated genes is likely to be in the effective elimination of intracellular pathogens. This was succinctly stated by Parkes et al[16]: "Taken together, the genetic evidence regarding *IRGM, ATG16L1, NOD2/CARD15,* and *IL23R* strongly implicates defects in innate immune pathways and handling of intracellular bacteria." Commensal organisms of the intestinal lumen are extracellular.

Immunologic studies of CD cells aim to identify common functional defects that are potentially linked to the lesions predicted by genetics. Stimulated by phenotypic similarities between CD and chronic granulomatous disease, the group of Tony Segal has conducted an intriguing series of studies looking for whether CD subjects share a common immune defect. Through studies of tissue repair at biopsy sites and recruitment of phagocytic cells at the site

of bacterial infection, this group has provided compelling evidence that CD patients manifest a primary immune deficiency of macrophages.[11,35] Further support for the importance of myeloid cells was recently reported in a study of bone-marrow chimeras derived from *Nod2−/−* mice. In a set of experiments aimed at understanding the role of NOD2 in IBD and graft-versus-host disease, Penack et al[36] found that the genotype of the myeloid cells transferred, and not the enterocytes, determined the response to enteric challenges. As phenotypic studies point to CD as a primary immunodeficiency of macrophages,[14] the finding that these same cells control intestinal inflammation in *Nod2*-disrupted mice argues that the primary defect in the pathogenesis of disease is manifest in macrophages, rather than epithelial cells.

RATIONALE FOR CD AS AN "INSIDE-OUT" DISEASE*

Intracellular bacteria are implicated in a number of infectious diseases, many of which follow enteric exposure to the pathogen. *Listeria monocytogenes* is a Gram-positive intracellular pathogen that causes disease weeks to months after a food-borne exposure. The process of infection has been worked out in exquisite detail, requiring the production of bacterial Internalin A that binds to human E-cadherin as a requisite step in the invasion of *Listeria* through epithelial cells.[37] Using mice that express human E-cadherin, Lecuit and colleagues[38,39] have been able to study the early cellular events of infection in great detail, observing that there is a remarkable paucity of acute pathology at the site of infection. Studies of the Gram-negative intracellular pathogen, *Salmonella,* also reveal that the process of invasion by the bacterium typically occurs without evidence of acute mucosal pathology, unless the model system is perturbed with the specific goal of enhancing mucositis.[40] In the case of mycobacteria, uptake of *M. avium paratuberculosis* after enteric infection in experimentally infected cattle also occurs in the absence of acute epithelial pathology.[41] In each of these examples, bacteria can be recovered from the mesenteric lymph nodes and liver within hours of infection, indicating that the establishment of infection is rapid, but does not require mucosal pathology. Given that different intracellular pathogens invade the gut via a pathology-negative process, a critical question is: "If intracellular bacteria do not cause mucosal pathology on the way in, do they cause pathology on the way out?" Indeed, when given intravenously, *M. tuberculosis* causes pulmonary disease. Likewise, intravenous infection with *M. avium paratuberculosis* leads to enteritis and fecal shedding.[42] The generation of mucosal pathology following intravenous exposure is clear evidence of an "inside-out" process.

Histopathologic analysis of CD tissue is consistent with a deep process that erodes "up" to the mucosa, potentially via the lymphatics. A chronic infection of the lymphatics is predicted to initially cause lymphadenitis, followed by cellular proliferation and fibrosis, eventually leading to lymphangietasia. These are the features of lymphatic filariasis due to *Wuchereria bancrofti,* where the characteristic state of elephantiasis results from obstruction of the inguinal lymph nodes. Remarkably, sclerosis of mesenteric lymphatics in pigs via formalin injection results in a disease with many of the features of CD.[43] Because lymphatic channels lack valves, a chronic infection can eventually spread in a retrograde manner, with microbes or their by-products being directed toward the tissue that the lymphatics originally drained. Thus, leg ulcers in a patient with elephantiasis do not represent a primary cutaneous process, but instead are the result of impaired lymphatic drainage from that limb. Analogously, the mucosal ulcer seen by endoscopy may represent a late event in the path to CD, even if it is an early diagnostic observation of the state of disease. Importantly, this model is supported by studies of postoperative disease recurrence, where it is reported that an impressive inflammatory infiltrate exists in the lamina propria at a time when only small aphthous ulcers are observed by endoscopy.[44] Extended further, in a subsequent report, Rutgeerts et al[45] stated: "Aphthous ulcers are found in the neoterminal ileum within weeks to months after operation and unidentified transmural lesions that lead to these mucosal defects probably occur almost immediately after the surgical procedure."

IMPLICATIONS OF CD AS AN "INSIDE-OUT" DISEASE*

In the model shown in Figure 1, the primary event is mucosal pathology followed by activation of the inflammasome. In contrast, for Figure 2 the central feature is mesenteric adenitis. This distinction may be critical for both the experimental models needed to optimally study CD and the hypotheses that guide these studies.

In a model where mucosal damage is primal, it follows that study of mucosal samples and animal models of mucositis will be rewarding. Indeed, studies have shown that mucosal biopsies are enriched for certain strains of *E. coli*, termed adherent-invasive *E. coli* that are able to associate with epithelial cells through interaction of their flagellin with the host adhesion molecule CEACAM-6.[46] It is not yet clear whether these bacteria initiate this event, or rather, whether these bacteria associate with damaged mucosa as a secondary phenomenon.[47] If the primary process instead occurs at a deeper site, then these mucosal bacteria may exploit mucosal defects to perpetuate the inflammatory pathology, itself a highly relevant observation. However, the absence of a primary etiologic agent may undermine

*[Correction made here after initial online publication].

the clinical utility of treatments directed against a superinfection. Therefore, while a number of investigators are conducting molecular bacteriologic analyses of mucosal biopsies using targeted[48] or metagenomic approaches,[49] these studies may be largely unrewarding if the disease occurs via an "inside-out" path. This alternative disease model predicts that a thorough search of deeper tissues, such as the submucosa, the lymph nodes, and the mesenteric fat, using modern tools such as in situ hybridization, in situ polymerase chain reaction (PCR), or tissue PCR, may generate an entirely different portrait of the microbes that are associated with disease.

The other problem with the existing model is that activation of the inflammasome is perceived to be an early event. Remarkably, this would occur despite the fact that mutations in *NOD2* lead to loss of bacterial recognition[29,30] and mutations in *ATG16L1* are associated with impaired autophagy.[50] Moreover, intracellular bacteria have evolved numerous strategies of infection that subvert host responses to enable their successful infection and persistence in the eukaryotic host. For instance, virulent mycobacteria actively prevent phagolysosome maturation, actively block apoptosis, and actively reduce cytokine production by macrophages.[51–53] Therefore, if the host defect involves impaired innate recognition of bacteria, and these organisms suppress host responses, it does not follow that acute activation of the inflammasome should occur. Because most small animal models aim to dissect an "outside-in" process, they provoke mucosal perturbations that result in acute inflammation within days of the exposure. To better understand a chronic process involving defective responses to intracellular pathogens, longer-term models are needed that reflect a biphasic process, involving impaired innate handling of the microbial challenge followed by subsequent inflammatory changes weeks to months later. For instance, studies of oral *Salmonella* infection describe a dynamic process; host immunity is critical for control of infection during a period of asymptomatic persistence but eventually the chronic infection leads to inflammation and fibrosis.[54,55] The same features are observed in chronic mycobacterial infections, such as pulmonary *M. tuberculosis* and *M. avium paratuberculosis* in livestock. These are not events that can be studied in 3–5 days.

The presented model for "inside-out" pathology invokes a lymph node-based disease, a specific anatomic prediction that may be proven incorrect. Nonetheless, there are a number of reasons to study the lymph nodes with particular care. First, radiologic studies show that the vast majority of CD patients have enlarged lymph nodes and that lymph node activity on magnetic resonance imaging (MRI) scanning is closely correlated with disease activity.[56] Whether these represent a primary or secondary process is not known, but nonetheless, enlarged lymph nodes are

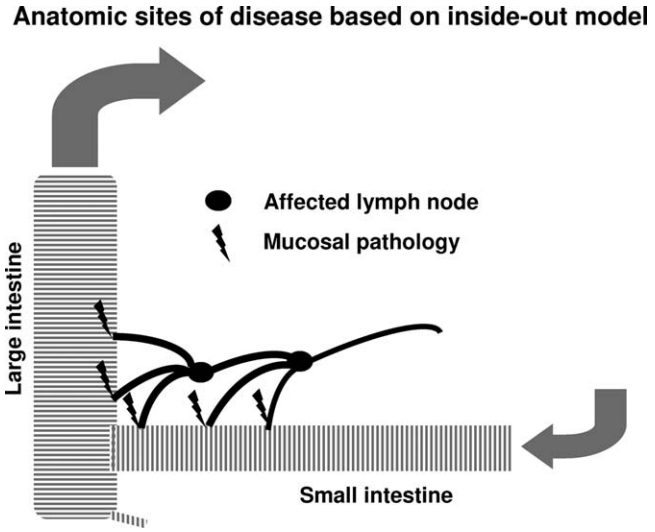

FIGURE 3. The alternative model predicts that mucosal pathology may result from retrograde spread of a deeper process via damaged lymphatic channels. In this example, disease in nodes of the superior mesenteric lymphatics would result in discontinuous mucosal lesions, between the middle part of the duodenum and the splenic flexure of the colon.

there and are accessible for study at surgical resection. Second, an ongoing inflammatory process in the lymph nodes might result in spill-over of bacteria or host effectors, potentially explaining the proliferating fat in the mesentery, where the nodes reside.[57] Third, a lymph node-based disease is consistent with skip lesions in the gut, in contrast to a superficial process, such as ulcerative colitis, that progresses across the mucosal surface (Fig. 3). Moreover, a lymph node-based disease in the superior mesenteric distribution could explain the transcendence of CD across small and large intestine, as >90% of CD lesions are found between the mid-duodenum and the splenic flexure of the colon. These are all hypotheses that can be tested, given the appropriate clinical samples and animal models.

The recent availability of banks of mucosal samples and high-throughput study methodologies such as expression profiling and proteomics together provide an attractive opportunity for clinical research on CD. Despite these resources, it may be that careful analysis of full-thickness surgical samples and the adjacent mesentery for the presence of intracellular pathogens would be more rewarding if one aims to identify bacterial agent(s) implicated in disease causation.[58] Likewise, experimental models of acute inflammatory pathology present an opportunity to dissect the processes involved in acute gut inflammation. However, to understand a disease characterized by proliferative changes and chronic inflammation, new experimental models may be required that reflect and build upon the latest

advances from phenotypic and genetic study of CD patients. It is remarkable that murine knockout models used to study IBD do not involve CD susceptibility genes, and that disruption of murine homologs of these genes does not result in chronic enteritis.

To best understand a chronic transmural and systemic disease, experimental models should contemplate the path to inflammation, as well as the state of pathology. While a purely "inside-out" model may not adequately explain the development of CD, the reliance on an "outside-in" model risks mistaking a state of inflammation that occurs in days with a state of inflammation that persists for decades.

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