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The immunopathogenesis of Crohn's disease: a three-stage model

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Abstract

The pathogenesis of Crohn's disease (CD) has remained an enigma for at least a century. There was considerable optimism that genetic linkage and genome-wide association (GWA) studies had identified genes causally responsible. However, the realisation that these genes make a relatively minor contribution to the development of CD has led to the acceptance of a 'missing heritability'. In contrast to the weak genetic effects, patients with CD almost without exception exhibit a gross phenotype, namely a profound systemic failure of the acute inflammatory response. This results in markedly delayed clearance of bacteria from the tissues, leading to local chronic granulomatous inflammation and compensatory adaptive immunological changes, as well as constitutional symptoms.

Introduction

'Inflammatory bowel disease' encompasses several distinct clinical entities, the most common being Crohn's disease (CD) and ulcerative colitis (UC). CD is a chronic, relapsing–remitting inflammatory condition predominantly affecting the terminal ileum and colon, associated with distinctive pathological features [1]. Unsuccessful attempts have been made to identify its cause since it first entered the literature at the turn of the 20th century.

Putative 'causes' have included infections with a variety of organisms such as mycobacteria, L-form bacteria, *E. coli* and measles virus. A host of other mechanisms have also been postulated, including auto-immunity and disordered T cell function [2].

The advent of molecular biological and gene sequencing technologies spawned studies relating disease phenotypes to particular regions of the genome and then to specific genes. This search appeared particularly fruitful in relation to CD where a number of statistically significant associations were identified.

Furthermore, gene targeting technologies produced mouse models of relevance to CD, either because the targeted gene corresponded to one of the associated genes described above, or its disruption predisposed to bowel inflammation interpreted as having some similarity to human 'inflammatory bowel disease'.

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Conflict of interest statement
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This review discusses the concept that the development of CD occurs in three stages. The first of these is ingress of bacteria and antigenic material into the bowel wall. In stage 2, a weak acute inflammatory response results in impaired clearance of this material. Chronic granulomatous inflammation and adaptive immune responses are subsequently provoked in stage 3, culminating in the development of CD. The potential relevance of recently identified susceptibility genes and the relationship of animal models to this pathogenic scheme will be evaluated.

Genetic studies and Crohn's disease

Twin and family studies confirmed a strong genetic influence on the acquisition of CD. For example, approximately 50% of monozygotic twins and 30% of offspring of two affected parents develop disease [3]. Linkage analysis and positional cloning strategies, together with subsequent genome-wide association (GWA) studies, have identified over 30 distinct genetic loci that confer susceptibility. Some of the most strongly associated genes included *CARD15*, the *IBD5* locus, the autophagy genes *ATG16L1* and *IRGM* and the IL-23 receptor [4•].

Whilst such studies have provided several important clues into genetic susceptibility and pathogenesis of CD, it must be stressed that all the polymorphisms identified to date cannot be regarded as 'causal'. The variants have very low penetrance, even in the case of *CARD15*. In a random sample of 100 000 people, an estimated 15 000 would be heterozygous for one *CARD15* variant, with around 500 homozygous or compound heterozygous [5]. Approximately 100 in the whole sample will develop CD, but only 28 of these patients will be simple homozygous or compound heterozygous for *CARD15* polymorphisms [6]. A similar situation exists for *ATG16L1*, but with even weaker effects.

It is increasingly apparent that GWA studies do not provide a comprehensive description of the entire genetic risk for any one condition. This is illustrated by three recent studies that sought to identify genetic factors determining human height (a classic polygenic trait with a strong genetic component), using data from approximately 63 000 individuals [7–9]. Whilst more than 40 important genes were identified, they accounted for just over 5% of normal height variation. A similar situation exists in CD; it is estimated that all the genes identified so far account for less than 20% of the total genetic risk [4•]. This has led to the recent suggestion of 'missing heritability' — that both the individual and cumulative effects of susceptibility genes identified to date are very small, and far less than the total heritability [10••].

The unifying findings in all CD patients are therefore phenotypic abnormalities, rather than defects in single genes. Polymorphisms in the identified genes, and many more that are as yet undiscovered, may confer susceptibility by contributing to mucosal barrier dysfunction, the innate immunodeficiency state or by influencing the propagation of chronic inflammation in the tertiary phase of the disease.

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Animal models and Crohn's disease

Numerous animal models of inflammatory bowel disease have been described, including chemically induced colitides, inbred animal strains, knockout or transgenic animals and adoptive transfer models [11,12]. These have been useful tools to investigate the physiology of bowel inflammation, highlighting roles for immune–microflora interactions. However, the direct applicability of most to human CD is unclear. Firstly, there are striking differences in pathological features, with few models demonstrating granuloma formation, discontinuous transmural inflammation and extraintestinal manifestations. Another limitation is the short lifespan of the mouse, which typically does not exceed four years. Given that onset of CD in humans peaks in the second and third decade, these models may well not adequately take into account the time dependence for the development of lesions. Furthermore, GWA studies have failed to demonstrate significant associations between many of the manipulated genes in animal models and human CD. Indeed, even between different animal strains, the effects of certain genetic alterations and exogenous manipulations are variable [11].

Conversely, animals with targeted manipulations of many of the recently identified 'CD susceptibility genes' do not develop a clear CD phenotype, in spite of immunological abnormalities. Macrophages isolated from *CARD15* knockout mice secrete reduced IL-12 in response to MDP stimulation, but do not develop spontaneous bowel inflammation [13]. Spontaneous colitis is likewise not observed in mice with a targeted deletion of *ATG16L1* in haematopoietic cells, nor in animals hypomorphic for *ATG16L1* expression, despite demonstrating increased susceptibility to acute DSS colitis, enhanced macrophage IL-1β secretion after LPS stimulation [14] and abnormalities in Paneth cells [15].

Therefore, in the absence of an obvious pathogenesis of CD, we propose a three-stage mechanism for the development of CD lesions (illustrated in Figure 1). This takes into account and integrates much of the existing knowledge of CD, including genetic factors and data from animal models.

Stage 1: penetration of luminal contents into the bowel wall

The development of CD is dependent on bowel contents. Diversion of the faecal stream in such patients is associated with the induction of remission [16,17], and experimental re-introduction of ileostomy effluent into the excluded ileum results in recurrence [18]. To produce CD lesions, components of the faecal stream must penetrate the intestinal barrier and gain access to the underlying bowel tissues.

The gastrointestinal epithelial cells normally form a relatively impermeable physical barrier to luminal contents, facilitated by tight junctions that impede paracellular transport [19]. A thick layer of mucus, largely composed of mucin glycoproteins, overlies the epithelial cell layer. Defensins, immunoglobulins and various other molecules are also contained within this mucus layer, which form an additional defensive component. These substances are secreted by enterocytes, lymphocytes, goblet cells and Paneth cells (the last being generally restricted to the crypts of Lieberkuhn in the small bowel) [20].

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Several studies have reported increased intestinal permeability in CD patients [21–24]. The underlying mechanism remains debated, as does whether it is a primary phenomenon or secondary to a chronic inflammatory state in the bowel. Although many studies investigated patients with active CD, increased permeability has also been reported in macroscopically normal small bowel of patients [25], which may predict recurrence [26]. These observations are compatible with the hypothesis that increased permeability is a primary initiating factor in CD.

Subsequent studies demonstrated increased permeability in a proportion of both healthy first-degree relatives [27] and spouses [28] of CD patients, arguing in favour of both environmental and genetic determinants. The underlying bowel permeability would make the mucosa more susceptible to damage. Whereas in most cases penetration of foreign material probably follows viral or bacterial infection, clearly a less robust mucosa will have reduced resistance. Other environmental factors such as trauma, high intra-luminal pressure, non-steroidal anti-inflammatory drugs and hypoxia could also play roles in causing mucosal damage.

Polymorphisms in mucin genes such as *MUC19* are associated with increased risk of CD [4•]; these variants could foreseeably increase the susceptibility of the mucosal barrier to injury. Alterations in expression and post-translational modifications of mucins have also been reported in CD [29]. Another CD susceptibility locus is located in a gene desert region on chromosome 5p13.1, which may be associated with altered expression of the prostaglandin E4 receptor (*PTGE4R*) [30]. *PTGE4R* may have roles in maintenance of mucosal barrier integrity, which is reflected in the increased susceptibility of *PTGE4R* knockout mice to dextran sodium sulphate (DSS) induced colitis [31].

Several mouse models underscore the importance of mucosal barrier dysfunction in the induction of bowel inflammation. Transgenic mice manipulated to express a dominant negative N-cadherin, a junctional adhesion protein, develop spontaneous inflammatory bowel disease [32]. Furthermore, introduction of mutations in the mucin encoding gene *MUC2* also results in spontaneous colitis, although the histopathological features are more reminiscent of UC [33]. Interestingly, the inbred SAMP1/Yit (Samp) mouse strain displays increased ileal permeability, which precedes the onset of a spontaneous ileitis [34]. In this model, the inflammation described is not dissimilar to human CD, with discontinuous, transmural leukocytic infiltrates and coalescence of macrophages into aggregates.

Stage 2: impaired clearance of foreign material from the bowel wall

It was realised over three decades ago that the consequences of the penetration of faecal contents into the underlying tissues would depend upon the adequacy of the acute inflammatory response. In CD this response is defective as the result of a primary failure of acute inflammation [35••]. Neutrophil accumulation to ‘skin windows’, as well as traumatised bowel mucosa, is impaired in CD patients, as a consequence of diminished concentrations of acute inflammatory mediators such as IL-8 and IL-1β at these sites [36••]. The neutrophils themselves function normally *in vitro* [37], and the addition of exogenous IL-8 to skin windows of CD patients corrected neutrophil influx to normal levels [36••].

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Pro-inflammatory cytokines are primarily secreted by resident tissue macrophages [38], which in the bowel are derived and continually replenished from peripheral blood monocytes [39]. Dramatically impaired secretion of pro-inflammatory cytokines by macrophages from CD patients was observed in response to stimulation with heat-killed *E. coli* [67••]. Subsequent experiments, including microarray analysis of the transcriptome in these cells, indicated defects in macrophage secretory systems as the underlying cause of this phenomenon, which results in mistargeting of cytokines to lysosomal compartments.

In contrast to previous studies, many of which describe elevated cytokine levels in biopsies or peripheral blood mononuclear cells (PBMCs) from CD patients, in our studies samples were obtained from patients with quiescent disease. Pure cultures of a single cell type (macrophages) were obtained, and their responses to defined stimuli examined. It is therefore improbable that the results obtained were secondary to a chronic inflammatory state in the bowel, further bolstered by the distinct cytokine profiles observed from macrophages cultured from UC patients.

It was hypothesised that delayed neutrophil recruitment would impair clearance of intestinal contents gaining access to the bowel tissues. We have recently been able to test this experimentally by injecting heat-killed *E. coli*, a gram negative coliform, into the subcutaneous tissues of the forearm. The accumulation of ^{111}indium labelled neutrophils to these sites was markedly delayed in CD compared to healthy controls. To determine whether this affected clearance of bacteria, the *E. coli* were labelled with ^{32}P, and the rate of disappearance of radioactivity from injection sites determined. Clearance was dramatically lower in CD, requiring, on average, 40 days compared to 10 days in healthy controls [67••].

In certain scenarios, the ability of neutrophils to degrade and remove the bacteria and other bowel contents might be compromised as a result of an inherited defect. Such a situation exists in patients with congenital, monogenic disorders of phagocyte function. Patients with chronic granulomatous disease (CGD), an immunodeficiency disorder caused by mutations in NADPH oxidase, frequently suffer from bowel inflammation that is indistinguishable from CD [40]. The cellular defect in this condition is not limited to bacterial killing — digestion is also severely impaired as a consequence of abnormal pH and charge compensation [41]. This therefore supports the hypothesis that clearance of foreign material is a critical factor in the development of CD.

Strong associations between other monogenic disorders of neutrophil function and CD have also been highlighted in recent reviews [42,43]. Mutations in single genes result in failure of neutrophil production and accumulation (congenital neutropenias and leukocyte adhesion deficiency), impaired digestion (CGD and Glycogen Storage Disease-1b) and defective phagolysosomal fusion and vesicle trafficking (Chediak–Higashi and Hermansky–Pudlak syndrome). As a consequence of these defects, these patients also have a highly impaired ability to clear foreign material and, as would be expected, have an increased susceptibility to CD.

The immunodeficiency phenotype is also consistent with findings from genetic studies. *CARD15* was the first CD susceptibility locus identified. It encodes NOD2, which is

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expressed in mononuclear phagocytes, epithelial cells and Paneth cells [44,45]. It is thought to interact with MDP, a constituent of bacterial cell walls, leading to activation of the transcription factor NF-kB and induction of pro-inflammatory cytokines. Polymorphisms in the leucine rich repeat domain predispose to CD, and are associated with reduced pro-inflammatory cytokine responses to MDP [36••,46]. *In vivo*, addition of exogenous MDP was shown to correct the defect in neutrophil accumulation in CD patients wild-type for *CARD15*, an ability that was abrogated by the presence of CD-associated polymorphisms. This suggests a possible 'compensatory' role for NOD2 in boosting weak acute inflammatory responses to bacteria [36••]. *CARD15* sequence variants may also be associated with reduced alpha defensin expression by Paneth cells [47], and altered anti-inflammatory cytokine production by PBMCs [48], suggesting that NOD2 could exert effects in multiple stages of CD pathogenesis.

GWA studies have also identified variants in the *ATG16L1* and *IRGM* genes that are associated with CD [49•,50•,51]. CD-associated mutations in *ATG16L1*, as well as functional knockdown of *ATG16L1* or *IRGM*, results in impaired autophagy and elimination of intracellular pathogens [52,53], in keeping with the concept that clearance of foreign material is important in the development of bowel inflammation.

There are few animal models that convincingly describe bowel inflammation occurring in conjunction with innate immunodeficiency. Mice with targeted deletions of *STAT3* in the bone marrow are a possible exception, where impaired innate immune function, including reduced NADPH oxidase activity, was demonstrated. Interestingly, these mice had histopathological features reminiscent of CD, with transmural inflammation and granuloma formation [54].

If CD arises from a systemic defect in innate immunity, one might expect patients to manifest increased susceptibility to bacterial infection. This may indeed be the case, and several studies have reported increased incidences of acute gastroenteritis [55] and urinary tract infection [56]. Larger scale studies are required to confirm this finding, which must take into account important confounding factors such as surgery, malnutrition and use of immunosuppressant therapy. Another consideration is bacterial load: most acute infections arise from multiplication of a small number of initial inoculating organisms, which even the partially attenuated innate immune response in CD might be sufficient to control. In contrast, the terminal ileum and colon, the sites most commonly affected in CD, contain large number of bacteria (approximately 10^8/g and 10^{11}/g, respectively [57]) which could foreseeably 'overwhelm' the impaired clearance mechanisms.

Stage 3: compensatory adaptive immune responses

In the absence of adequate neutrophil recruitment, the remaining uncleared debris will be phagocytosed by macrophages. These cells subsequently form granulomata, in an attempt to contain this material. Macrophage activation will then result in a 'second wave' of secretion of pro-inflammatory cytokines and chemokines that will drive recruitment of T cells to the site, as well as their polarisation to the characteristic Th1 phenotype. This phase of chronic inflammation is temporally distinct (occurring days to weeks after penetration) from the

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initial acute inflammatory response described in stage 2 (which occurs within several hours). At this stage, even if net production of cytokines by each cell were lower than normal, the overall number of cells will be so great that damaging concentrations of cytokines will be produced. Local tissue damage, as well as systemic responses then ensue, giving rise to the symptoms of CD.

In this model, granuloma formation and lymphocytic infiltration represent compensatory mechanisms for the initial failure of acute inflammation, rather than primary pathogenic defects. In support of this, CD does not fulfil Witebsky's postulates for defining an autoimmune condition [58]. Furthermore, whilst there have been reports of autoreactive T cells and autoantibodies in CD patients, their mechanistic relevance has not been demonstrated. These may therefore be generated secondary to a chronic inflammatory state in the bowel, characterised by a high degree of cell turnover and presentation of antigens to the adaptive immune system.

The development and perpetuation of the chronic inflammatory response will involve products of variety of different genes, which could further contribute to CD susceptibility. For example, polymorphisms in genes encoding the IL-23 receptor, and the IL-12B subunit are associated with CD, with the minor alleles conferring protection [4•,59]. The proteins encoded by these genes may modify the chronic inflammatory response to bacteria through the induction of Th17 cells [60], effects on immunosuppressive T-reg cells [61], driving granuloma formation [62] or by other mechanisms. The association of these genes with other chronic inflammatory conditions, including UC [63], is noteworthy.

Despite having a secondary role in disease pathogenesis, the chronic inflammatory response is nevertheless important clinically, being responsible for the local lesions such as ulceration, fistulation and stricturing, as well as systemic symptoms. Immunosuppressive therapies and drugs directed against TNFα target this stage of disease pathogenesis by direct cytokine blockade, induction of leukocyte apoptosis [64] and stimulation of T-reg cells [65]. Whilst suppressing chronic inflammation in the final stage of CD pathogenesis, thereby inducing remission, therapies such as corticosteroids are not efficacious at preventing relapse [66], and may indeed exacerbate the underlying innate immunodeficiency.

Conclusion

In conclusion, we propose a model for the pathogenesis of CD that involves three temporally distinct stages: penetration of faecal contents into the underlying tissues (stage 1), which is followed by a weak acute inflammatory response to this material that arises from a generalised defect in pro-inflammatory cytokine secretion by macrophages. The diminished neutrophil recruitment that occurs as a consequence of this defect results in impaired clearance of antigenic material (stage 2). Compensatory responses will then come into play (stage 3), which includes the recruitment of T cells and their polarisation to a Th1 phenotype. The model is consistent with findings from recent genetic studies, as well as animal models of inflammatory bowel disease.

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This scheme of CD pathogenesis has significant implications, both in terms of future research and therapies. It identifies an immediate need to understand the mechanisms of cytokine trafficking within immune cells, and locating variants in these processes that may contribute to the development of CD. More fundamentally, the phenotype that appears to predispose to CD is one of an impotent acute inflammatory response. The magnitude of the latter will be determined by the collective effects of a very large number of different gene products and environmental factors. A bioinformatics approach will be required to understand how these inflammatory networks interact, the individual factors summate, and the combinations that predispose to CD.

This model highlights the value of a ‘phenotype to gene’ approach to CD research. Further functional characterisation of the macrophage phenotype and mucosal barrier function, as well as discovering the underlying cellular and molecular mechanisms, will help elucidate the identity of the genes that make up the ‘missing heritability’, which are likely to be of low effect size and penetrance. Prerequisites to any further studies will be precise clinical phenotyping of patient groups (not using a generic ‘inflammatory bowel disease’ cohort), and careful distinction between primary phenomena and those occurring secondary to chronic inflammation or its treatment. The model will also be useful in evaluation of future candidate susceptibility genes identified in GWA and familial studies. Given the complex, polygenic nature of CD, the precise molecular lesions will differ between patients and may well be impractical to correct clinically. However, therapies directed at protection of the mucosal barrier, and stimulating the acute inflammatory response may prove efficacious for the prevention of relapse in patients with quiescent disease. In a wider context, this model of CD pathogenesis may be equally applicable to many other chronic inflammatory conditions.

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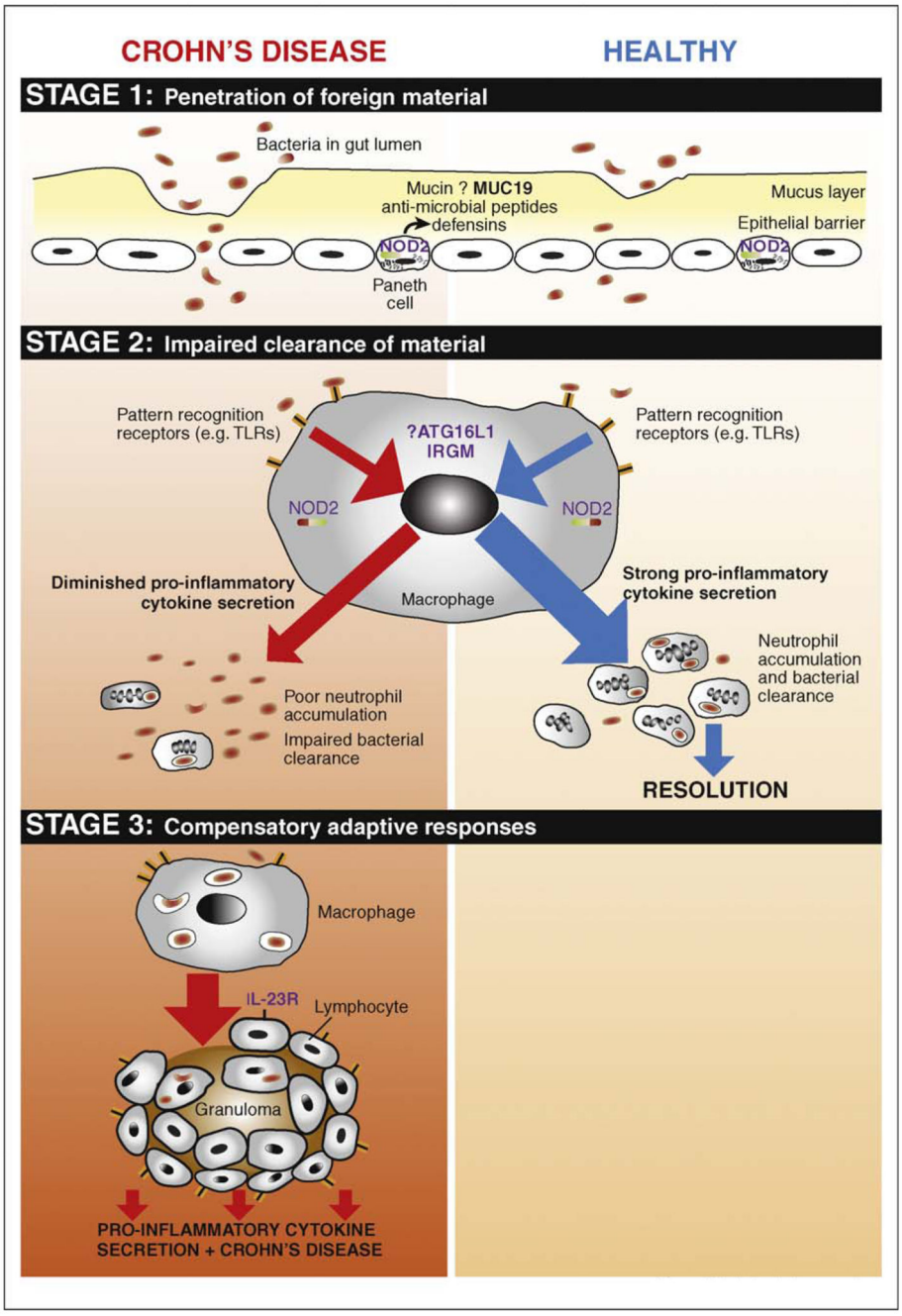

Figure 1.

The immunopathogenesis of CD occurs in three temporally distinct stages. Penetration of luminal contents into underlying tissues occurs in stage 1, which may be facilitated by environmental factors such as infection, or inherent defects in the mucosal barrier. In healthy individuals, resident macrophages secrete pro-inflammatory cytokines in response to this material, resulting in neutrophil accumulation, clearance of the material, and thereby resolution. In CD patients, defective secretion of pro-inflammatory cytokines by macrophages results in impaired neutrophil influx and clearance of foreign material (stage

2). Subsequently, chronic inflammatory responses (stage 3) will be triggered, giving rise to the characteristic features of the CD lesion.

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