The pathogenesis of Crohn’s disease in the 21st century

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Summary
Ten years ago Crohn’s disease remained a highly complex disorder with an unknown aetiology and a poorly understood pathogenesis. Research in this field had repeatedly confirmed the presence of an activated intestinal immune system but the factors underlying this had not been identified. Since then, the significance of genetic susceptibility has been established and underlined by the discovery of the NOD2 gene. In turn, this has gone on to signal the importance of the innate immune system and the critical relationship between the gut flora and the intestinal mucosa. These factors, together with independent environmental triggers such as cigarette smoking, form the basis of Crohn’s disease pathogenesis in the new century.

Key words: Genetic susceptibility, NOD2, innate immunity, gut flora, intestinal interfaces, smoking, appendicectomy.

INTRODUCTION
Crohn’s disease (CD) remains one of the major challenges in luminal gastroenterology. This disorder is characterised by a chronic, transmural inflammatory process which can affect any part of the gastrointestinal tract. The natural history of the disease is variable, but the majority of patients tend to have a relapsing and remitting course. The prevalence of the disease is close to 0.2% in developed nations, and can appear in both children and adults with the peak age of presentation in the third decade of life.1 Morbidity relates to the chronic symptoms of diarrhoea, abdominal pain and malnutrition which have a significant impact on a patient’s quality of life.2 The majority of patients require medication, with up to 50% on immunosuppressive therapy in specialist-based cohorts, and a similar number of patients requiring intestinal resection within 5 years after diagnosis.3,4

The aetiology of CD remains to be precisely defined but the current favoured hypothesis describes a genetically susceptible individual who encounters a single or series of environmental triggers to manifest the disease. The key elements in this hypothesis have received much support from the recent description of the first susceptibility gene for CD called NOD2/CARD15 and its proposed mechanism of action.5,6 One of the major ‘environmental’ driving forces in the inflammatory process are the commensal intestinal bacteria, as demonstrated by work with animal models and from clinical observations in CD.7 Manipulation of the gut flora is currently undergoing extensive investigation as a therapeutic manoeuvre to modify the antigenic stimulus and hence the local immune response in CD. Other environmental factors capable of influencing the course of the aberrant immune response in CD include cigarette smoking8 and, more controversially, early appendicectomy.9 In this review, we will concentrate on recent advances in our knowledge of the genetics of CD, on the interplay between intestinal bacteria and the innate immune response, and on those studies that have been replicated by independent data sets.

GENETIC SUSCEPTIBILITY
The role of genetic susceptibility in the pathogenesis of CD was long supported by familial clustering of the disease.10 Furthermore, the concordance rate between monozygotic twins (44%) was found to be significantly higher than in dizygotic twins (3.8%).11 In contrast to classical single gene disorders, carriage of multiple genes is likely to contribute to disease susceptibility and the presence of one abnormal gene will not necessarily result in the CD phenotype. In addition, similar phenotypes may be determined by differing genetic profiles.

Reports on the association between polymorphisms in the NOD2/CARD15 gene and susceptibility to CD have improved our understanding of CD pathogenesis, and led to a flurry of further research activity both in molecular genetics and immunology. NOD2/CARD15 encodes a cytosolic protein expressed primarily in monocytes. It contains two N-terminal caspase recruitment domains, a central nucleotide-binding domain and a C-terminal regulatory domain containing leucine-rich repeats.12 It has structural homology to the apoptosis regulators Apaf1 and Ced4 as well as a class of plant cytosolic disease resistance (R) gene products.13 Overexpression of NOD2/CARD15 enhances caspase-9-induced cell death and induces NF-κB activation, a transcription factor central to the expression of inflammatory cytokines in CD. NF-κB has previously been recognised to be activated in mononuclear cells in the lamina propria in CD14 and inhibition of NF-κB by glucocorticoids and sulphasalazine has long been employed in the treatment of CD.

Three polymorphisms in the NOD2/CARD15 gene have been positively associated with CD, two single nucleotide polymorphisms (Arg702Trp, Gly908Arg) and a frameshift mutation (Leu1007fsinsC).5,6,15 All are located in or near the leucine-rich repeat C-terminal regulatory domain critical for the recognition of bacterial components.6 This observation clearly supports the concept that dysregulation of the innate immune response via a mutant monocyte cytosolic receptor for bacterial components contributes to CD
pathogenesis. Functional studies performed to date have involved transfection of cells with wild-type and mutant NOD2 plasmids. Response to bacterial lipopolysaccharide as measured by NF-κB activation was, perhaps surprisingly, significantly reduced with the mutant protein.5 This suggests that the role of the mutation in disease pathogenesis is not as simple as expected. Rather than simply causing an exaggerated inflammatory response to bacterial components, it may alter the transcription of down-regulatory factors such as interleukin-10. Alternatively, hypo-responsiveness of the protein conferred by mutation may alter the mucosal immune system’s tolerance to luminal bacteria early in life. However, the early transfection studies have used a limited number of bacteria, and omitted the Gram-negative organism, *Escherichia coli*. Further, more extensive analysis therefore may reveal a spectrum of NF-κB activity when the mutated protein comes into contact with human intestinal bacteria. Finally, it is important to consider the role of the mutated NOD2 protein in monocyte cell death. Previous studies in CD have already provided evidence supporting an interruption to normal activation-induced cell death in CD intestinal T cells.16,17 If this abnormality were to include gut monocytes due to the NOD2 mutations, then this may be a plausible explanation for both novel and recurrent inflammation in CD.

Carriage of a mutation in the NOD2 gene alone is not always adequate for disease development as one might expect in a polygenic disorder. Carriage of only one mutant allele confers a 1.5–3-fold increased relative risk of developing CD, whereas carriage of two mutant alleles results in an 18–44-fold increased risk compared with wild-type.5,6,15 In contrast, Esters *et al.* have demonstrated a similar mutant allele frequency in the unaffected siblings of CD patients.18 This supports the hypothesis that a single gene mutation may be insufficient to manifest disease phenotype, and interaction with other genes as well as environmental factors is necessary. Some important associations have already been demonstrated between the two major CARD15/NOD2 single nucleotide polymorphisms [SNP8 (Arg702Trp) and SNP13 (Leu1007fsinsC)] and disease phenotype. Firstly, these mutations do not confer susceptibility to ulcerative colitis (UC), suggesting an alternative inciting event for gut inflammation in CD compared with UC.5,6,15 With respect to CD, the statistically significant independent associations are with younger age at diagnosis and with ileal disease.19,20 One of the early studies also shows an independent association with structuring disease.20 and another demonstrates that the NOD2 mutation independently protects against fistulising CD.19 An independent Australian study supports the associations with disease site and behaviour (Eri R, Hume G, Purdie DM, *et al.* CARD15/NOD2 genotype-phenotype correlation in an Australian Crohn’s disease population, in preparation.). Racial differences in allele frequency have also been clearly demonstrated. A study of Japanese CD patients by Inoue *et al.*, failed to show any significant association between the three major NOD2/CARD15 SNPs and CD and a novel mutation has been identified in significant association with an African-American CD population.22 However, description of phenotype and patient selection remain problematic, making it difficult to compare these recent studies with each other. Currently, it is perhaps best to use the Vienna classification for CD, which was based upon a large international collaboration.23

### AFTER NOD2 – OTHER CD SUSCEPTIBILITY LOCI

With increasing work carried out on the NOD2 gene, it is becoming clear that variants of this gene will only account for 20–30% of Crohn’s disease.20 Other major susceptibility loci have been identified on chromosomes 12 (IBD2), 6 (IBD3), 14 (IBD4), and 5 (IBD5). The original identification of IBD2 was made by Satsangi *et al.*, using 186 affected sibling pairs in 160 UK families.24 The strongest linkage was found in a region on the long arm of chromosome 12, and specifically with the marker D12S83. Replicative studies have confirmed this linkage and one in particular has identified that this locus is most likely involved in UC pathogenesis but not CD.25

As with IBD2, work on IBD3 which includes the major histocompatibility complex, has predominantly found associations with UC rather than CD. Work in this area has been hindered by the complexity and polymorphic nature of this region, with significant associations sometimes confounded by linkage disequilibrium and the inability to reproduce initial results using independent datasets. Specific HLA haplotypes on 6p (located in the region of IBD3) have been associated with different inflammatory and autoimmune diseases. HLA molecules are critical to antigen presentation to T lymphocytes, hence forming another link between the environment, the innate and the adaptive immune responses. The polymorphic genes HLA-A, -B and -C encode class I molecules whereas class II molecules are encoded by HLA-DR, -DQ and -DP. A recent meta-analysis suggested that DR7, DRB3*0301 and DQ4 are positively associated with CD, compared with DR3 and DR2 which have a negative association with the disease.26 Other single associations may have been underestimated, as haplotypes in this region are highly conserved and, therefore, the primary variable is more difficult to determine. Ahmad *et al.* recently reported their further analysis of the HLA region and CD.27 Disease susceptibility was confirmed with DRB1*0701, whereas DRB1*1501 was again found to be protective. A novel association was found with Cw*0802. The study was extended to look for an association with disease phenotype. HLA-DRB1*0701 was significantly associated with the presence of ileal disease when compared with controls. In contrast, the ‘autoimmune’ HLA haplotype, A1-B8-DR3, was associated with the presence of colonic disease.

The other major area of interest in this region has been the tumour necrosis factor-α (TNF-α) gene, a strong pathogenetic and positional candidate. TNF-α influences a number of key pathways including the immuno-inflammatory response (through immune cell activation, cell death and cell trafficking), and tissue remodelling and repair (by inhibiting collagen synthesis and increased production of tissue metalloproteinases)28–30. Its role in CD has recently been strengthened by the results of treatment with the anti-TNF drug, infliximab, and from both European and Japanese studies which have linked the gene to disease susceptibility and disease behaviour.31 These studies are also supported by the functional significance of some of the TNF-α promoter polymorphisms, including those at positions −238, −308, −863, −857 and −1031, which show significant differences in circulating levels of TNF-α protein, depending on the inherited alleles at those positions.32 However, as alluded to above, there have been
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INTESTINAL BACTERIA

The CARD15 gene has provided researchers with a framework to help achieve these goals. Although a single causative mutation has not been identified in this region, inheritance of a specific haplotype of these cytokine genes is thought to be responsible for disease susceptibility. SNPs may affect the regulation of one or more genes, particularly the immunoregulatory cytokines, IL-4, IL-5 and IL-13, which have an important role in the direction of the immune response based upon the Th1/Th2 paradigm. Armuzzi et al. have since reported an association between IBD5 and perianal CD as well as ileal disease in patients not carrying the NOD2/CARD15 Leu1007fsinsC variant (SNP13).

Several other regions have been identified in association with CD in studies carried out in Europe, Canada, Australia and the US. However, extensive work is still required to refine these regions to make gene identification a more realistic target. The successful identification of the NOD2/CARD15 gene has provided researchers with a framework to help achieve these goals.

INTESTINAL BACTERIA

The important link between genetic susceptibility and inflammation is likely to be mediated by intestinal bacteria. The hypothesis that a single pathogen may be responsible for this has been superseded by a key role for the local commensal bacteria. This group represents an enormous antigenic reservoir, with over 10^14 organisms from at least 500 species. Appreciation of the diversity of these bacteria has only become possible with the increasing use of molecular probes as previous culture techniques revealed less than 50% of the total number of species. The role of these bacteria in helping to shape the intestinal mucosa and its immunoinflammatory response has predominantly come from work on animal models. Studies on animals kept under germ-free conditions indicate that the gut mucosa remains underdeveloped with reduced lymphoid tissue, epithelial proliferation and motility, compared with colonised animals. Commensal bacteria prime the immune response resulting in a state of ‘physiological’ inflammation, with further fine-tuning of the B and T cell repertoire and the cytokine balance with exposure to an increasing array of organisms in early life.

A change in the host genetic background can disturb this controlled inflammation as clearly demonstrated in an increasing variety of animal models of IBD. These may develop the disease spontaneously as in the Samp/Yit and C3H/HeBir mice, or following physical (haptent administration, adoptive transfer) or molecular manipulation (transgenes/knockouts). In all of these examples, expression of disease depends upon intestinal colonisation with bacteria suggesting that ‘pathogenic’ bacteria are not required for disease to occur but commensals may drive the inflammatory response in susceptible individuals. Similarly, intestinal bacteria have been shown to play an important role in CD, with diversion of the faecal stream and antibiotics both being used as effective therapeutic manoeuvres to control inflammation, and recurrence of disease being demonstrated upon re-exposure of excluded bowel to intestinal contents. There also appears to be a spectrum of inflammatory activity that can be generated by different species of intestinal bacteria, with caecal bacteria producing a more severe inflammatory response compared with certain lactobacilli which do not produce inflammation. Collectively, animal models of IBD also indicate that similar phenotypes may be acquired from different genetic backgrounds and in association with a wide variety of abnormalities within immune and non-immune intestinal compartments.

THE HOST-BACTERIAL INTERFACE

Intestinal bacteria help to shape the structure and function of the gut mucosa. Therefore, investigation of the host–bacterial interface is essential in understanding mechanisms involved in the tight regulation and coordination of non-immune, innate and adaptive immune responses to luminal antigens. The ‘epithelial defence’ includes the glycocalyx, tight junctions between epithelial cells and gram quantities of mucus. In addition, recent studies have identified a number of antimicrobial peptides including bactericidal permeability-increasing protein (BPI), lactoferrin, lysozyme and the β-defensins.

The epithelium and subepithelial layers also act together as a ‘sensory’ organ by continuously monitoring and sampling the intestinal contents. This involves a number of pattern recognition or Toll-like receptors (TLRs) which are now thought to be key regulators of the innate immune response. TLR-2 binds peptidoglycan and other bacterial lipoproteins that trigger different arms of the mucosal immune response including cytokine production and B cell activation. In addition, TLR-2 signals for both cell activation and apoptosis, a property shared by tumour necrosis factor receptor-1 (TNFR1), and potentially essential for the resolution of the immunoinflammatory response. TLR-4 binds lipopolysaccharide together with CD14 and by internalisation prevents inappropriate NF-κB activation. These TLRs are characterised by an extracellular leucine-rich repeat domain (as seen in the NOD2 molecule), and an interleukin-1 receptor type 1-like intracellular signalling domain. Recent studies indicate that intestinal macrophages do not express TLRs in the absence of inflammation and hence are unable to produce pro-inflammatory cytokines such as IL-1. In the inflamed state, such as in CD, TLR-2 and -4 are expressed, the cells are able to bind LPS and then produce high levels of IL-1.

This study did not measure macrophage apoptosis after LPS-induced activation which is an important omission. As indicated above, activation and apoptosis of mucosal immune cells go ‘hand-in-hand’ in terms of controlling both the initiation and the resolution of an inflammatory response. Prolonged macrophage survival after activation through the TLR pathway may lead to an inappropriately aggressive immune response, and by on-going production of Th1 cytokines such as IL-6, may also contribute to the resistance of T cell apoptosis seen in CD.
In addition to signals picked up by TLRs, the intestinal contents are continuously sampled by specialised epithelium (M cells), and by dendritic cells which use a series of dendrites that span epithelial cells without disrupting the tight junctions. Animal studies indicate that ‘normal’ luminal antigens will generate a Th2 and/or a Th3 mucosal immune response, including production of interleukin-4 (IL-4), IL-5, and IL-10 (Th2) and transforming growth factor-β (TGF-β) (Th3). IL-10 is an important ‘down-regulator’ of inflammation, while TGF-β plays a key role in the induction of isotype switching to IgA and in oral tolerance. However, human work using Peyer’s patch T cells taken from uninfamed ileal biopsies suggests that the Th1 route may be the default pathway, initially mediated by IL-12 secretion from these cells. Similarly, patients with CD also generate a predominant Th1 mucosal immune response to their gut bacteria, characterised by production of IL-12 by antigen-presenting cells (dendritic cells), IL-1, IL-6, and TNF-α by activated macrophages, and IL-18 and interferon-γ by activated T cells. Unlike the normal situation, the Th1 response in CD is then capable of triggering a series of pathological steps, including synthesis and release of activated metalloproteinasewhich mediate tissue destruction, and up-regulation of the local inflammatory response by induction of chemokines and adhesion molecules required for recruitment of additional effector cells.

What differentiates a controlled Th1 response in the normal individual from a response leading to uncontrolled inflammation may be found in those mechanisms that oppose the Th1 response itself. These include immuno-regulatory T cells that secrete inhibitors of inflammation such as IL-10 and TGF-β, and the process of activation-induced T cell death. Analysis of these separate pathways in CD may indicate possible defects. Resistance to the down-regulatory effects of IL-4 has been demonstrated in the mucosal mononuclear cell population of patients with CD, and a reproducible resistance to T cell apoptosis mediated by an IL-6/IL-6R signalling pathway has been identified in these patients. This may lead to a rapid accumulation of aggressive, pro-inflammatory mononuclear cells in an environment that is characterised by high antigenic load and a potentially vulnerable epithelium, thus setting the scene for a chronic inflammatory disorder.

Further investigation of this host–bacterial interface is essential, particularly with respect to CD. Identification of host genetic factors that may influence the response to commensal organisms, and a more detailed knowledge of differences in the bacteria that colonise a CD intestine compared with a normal intestine, will help scientists and clinicians to focus on those areas that may prove most therapeutically effective. In particular, probiotics may represent a sound therapeutic alternative in patients with CD, having been successfully used in patients with UC and pouchitis. Probiotics are live organisms that can influence the host–bacterial interface beneficially by competing with more pathogenic organisms at the epithelial surface, by switching the mucosal immune response from Th1-dominant to Th2, and by stimulating non-immune defence mechanisms such as increased mucus and short-chain fatty acid production. Probiotic bacteria include lactobacilli and bifidobacteria, but other strains such as E. coli have also been used, and are usually administered in the form of a capsule or powder. Taking this concept several steps further, Steidler et al. have genetically engineered a lactobacillus lactis species to secrete IL-10 which then demonstrated therapeutic efficacy in a murine model of IBD. Although there may be safety considerations with such a novel approach, the intragastric administration would have potentially significant advantages over the parenteral route that has been used in human trials of a number of biological agents for CD.

**SMOKING AND CD**

A number of discrete observations underline the importance of environmental factors other than intestinal bacteria in the pathogenesis of CD. These include the 44% concordance rate for CD in monozygotic twins, the dramatic increase in the frequency of the disease over the past 50 years, and the striking increase in both CD and UC seen in Asian and African families who have migrated to the UK over a similar time span. Although a number of these factors have been investigated, including prenatal events, breastfeeding, childhood illnesses, diet, hygiene, occupation, education, climate, pollution, stress, and the oral contraceptive pill, the most established association is with cigarette smoking. A number of independent case–control studies indicate that smoking is associated with a higher risk of developing the disease, while continued smoking significantly influences disease progression, with increases in clinical, endoscopic and surgical recurrence rates, and a reduction in patients’ quality of life. Retrospective studies have identified the closest association between smoking and CD as being with women and those patients with small bowel disease.

This interaction is made even more relevant to disease pathogenesis by the reciprocal relationship between smoking and UC. Independent studies demonstrate that UC is predominantly a disease of non-smokers, with the majority of these being ex-smokers. This has been supported by the modest beneficial effects of nicotine patches in the treatment of patients with mild UC. The role of smoking as a major factor in determining disease phenotype has also been explored in a large family study of 339 affected sibling pairs of whom 89 were discordant for smoking and 23 were discordant for disease. In 21 of 23 pairs discordant for disease, CD occurred in the smoker and UC occurred in the non-smoker (OR 10.5; 2.6–92; P < 0.0001). The effect of smoking on the development of CD was again strongest in women. Although the sib-pairs in this study were not identical, the strength of the association between smoking status and disease phenotype provides further supportive evidence for the role of cigarette smoking in the aetiology and pathogenesis of IBD.

Despite all these studies, our understanding of the mechanisms behind the association with smoking remains very poor. Studies have included the functional effects of smoking on circulating lymphocytes, macrophages and natural killer cells, and the effects of nicotine and smoking on both peripheral blood and mucosal cytokine levels. Work in animal models has implicated a neural pathway to explain the deleterious effect of nicotine administration on mucosal damage, but this has not been replicated in man. None of this work supports a coherent hypothesis to explain the reciprocal relationship of smoking with UC and CD. However, one pathway which appears to play an important
role in both diseases and which may be influenced by smoking is apoptosis. Several studies have now shown a resistance to T cell apoptosis in CD, mediated by IL-6 and the BCL-2 family of anti-apoptotic genes. While in UC there is a significant increase in epithelial cell apoptosis compared with normal epithelium. Smoking can decrease apoptosis in a number of cell lines, and this is mediated, at least in part, by the BCL-2 pathway. Therefore, an alternative hypothesis to help explain the relationship between smoking and IBD would implicate smoking in the resistance to apoptosis seen in the T cell population, and a positive role for smoking in the restitution of the diseased epithelium in UC by reducing epithelial cell death. In view of the very strong and replicated association with smoking, further studies are warranted to test this and other novel hypotheses.

APPENDICECTOMY AND CD

The appendix, despite being part of the mucosal immune system and essential to development of the primary antibody repertoire in the rabbit, was not considered relevant to IBD pathogenesis until recently. Multiple studies have now demonstrated a highly significant negative association between UC and appendectomy. The relationship with CD has been less clear, and this is thought to be due to the misdiagnosis of appendicitis in patients presenting with CD, and hence the removal of the appendix at the time of the original diagnosis of the disease. Although less common now, this misdiagnosis may be a significant confounding factor in retrospective studies of patients diagnosed 10–20 years ago, and this issue has been alluded to but not directly addressed in previous work. However, two recent studies have addressed this issue directly and find a similar significant negative association between prior appendicectomy and CD. One study goes on to examine the effects of prior appendicectomy on disease characteristics in IBD, showing a greater impact on the course of UC compared with CD. In the CD cohort, prior appendicectomy significantly delayed the age of presentation of the disease but had no effect on need for immunosuppressive therapy or surgery. In the UC cohort there were also significant reductions in the need for immunosuppressive therapy and colectomy. These observations raise a number of questions concerning the role of the appendix as part of the gut-associated lymphoid tissue (GALT) in humans, and the potential inverse relationship between appendicitis and IBD. Of further interest is the observation that rates of appendicectomy in first-degree relatives of patients with UC and CD are similar to their affected relatives, and significantly lower than matched controls. This once again raises the importance of genes versus environment in disease pathogenesis, whether related to the development of IBD or appendicitis. Further work on the appendix and appendicitis may help to identify novel risk factors for IBD related to both the host and to intestinal flora.

CONCLUSION

Following a period of ‘information accumulation’ but limited progress, the past few years have seen dramatic improvements in our understanding of the pathogenesis of CD. Some of the key developments that have led to this include a significant increase in our ability to unravel the genetics of complex diseases, a better understanding of the relationship between the host and commensal bacteria, and several epidemiological observations that underpin the importance of environmental factors in IBD.

The challenge that now faces both scientists and clinicians working in this field is the conversion of this new knowledge into therapeutic benefits for IBD patients. This may take the form of new genetic screening tools for establishing patients’ disease characteristics earlier and more reliably, similar tools to predict response to therapy, and modification of the host–bacterial interface with probiotics.

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