CHAPTER 13

Multiparameter Analysis of Immunogenetic Mechanisms in Clinical Diagnosis and Management of Inflammatory Bowel Disease

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Abstract

The integrity of the intestinal mucosa depends on a functional coordination of the epithelium, lumenal microorganisms, and the local immune system. The mammalian immune system is superbly organized for innate and adaptive recognition of microbial antigens,[1,2] a defensive capacity that must be balanced against the tissue damage produced by immune activity to preserve normal intestinal function.[3] Inflammatory bowel disease (IBD) is generally thought to reflect an impairment in this balance, due to a combination of host genetic traits that shift the balance of immune and epithelial function to commensal microbiota, and perhaps the composition or activity of certain microbial elements as well.

There has been much progress defining the fundamental disorders of these host traits, immunologic processes, and microbial targets in inflammatory bowel disease.[4] Other fields of clinical and geologic microbiology are teaching us about the dynamic interaction of commensal bacteria with their host environment.[5,6] These lines of investigation have revealed not only important insights about inflammatory bowel disease (IBD) pathogenesis, but also defined technologies and tools useful for its diagnosis and clinical management. This review focuses on these advances at the translational interface. We will first consider the innate anti-microbial response, centering on the utility of NOD2 genotyping for predicting disease susceptibility, prognosis, and therapeutic response profile. We will then turn to the adaptive anti-microbial response, focusing on the application of antibodies to fungal and bacterial species and products for Crohn's disease (CD) diagnosis and prognosis, and immunogenetics of T cell immunosuppression management. Finally, we will describe autoimmune mechanisms in IBD, with particular attention to autoantibodies in IBD diagnosis and infliximab responsiveness. We will conclude with the concept of multiparameter analysis of patients, to refine patient characterization and stratification in diagnosis and clinical management.

NOD2 and Innate Anti-Microbial Processes in IBD Diagnosis

During the past decade, a major effort by several laboratories in genome-wide analysis of at-risk human populations has culminated in the identification of several loci associated with CD and/or ulcerative (UC) susceptibility.[7] The greatest progress has occurred with the IBD1 locus on chromosome 16, which proved to reflect mutations in the NOD2/CARD15 gene.[8,9]

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This protein encodes an important innate immune microbial recogntion molecule—an intracellular peptidoglycan receptor which activates CARD and NF-κB dependent pathways of cellular activation.[10] NOD2 mutations primarily if not exclusively affect CD susceptibility, indicating that this protein pertains to the pathobiology of CD rather than UC. Several NOD2 alleles are associated with CD disease risk, and relate to loss-of-function missense mutations of the NOD2 protein.[11]

Several studies have shed light on the utility of NOD2 mutations as adjuncts to disease diagnosis. First, it should be noted that the overall genetic contribution to CD is estimated to be only a minority of disease risk (about 36%),[12] and NOD2 is only one of several loci contributing to this facet of disease pathobiology. Second, even among individuals with CD, most individuals are heterozygote for loss-of-function NOD2 alleles, which would be expected to confer lesser risk than the homozygote state. A practical issue is the number of pertinent disease-susceptibility alleles, since a large number of such alleles, each at low frequency, might additively be a large percent of genetic disease risk in the population. Fortunately, it so far appears that only three haplotypes account for most pertinent NOD2 mutations.[11,13]

With these considerations in mind, studies to this point indicate that the heterozygote state is associated with about 2-fold excess disease risk, and about 24-fold in the homozygote state.[13-15] Since the prevalence of CD is rather low in the population (about 0.1%), neither the heterozygote nor homozygote states of these NOD alleles are suitable as screening diagnostic criteria for CD. However, the allelic state may be useful in validating diagnosis in patients in combination with other independent parameters (clinical, immunologic, or other susceptibility genes) permitting disease stratification (see below).

A more immediate use of NOD2 allelism is for disease phenotype and prognosis. Patterns of CD clinical phenotype are substantially hereditary,[16] and several recent studies concur that NOD2 mutations are an important predictor of fibrostenosing ileal disease.[13-15] Such information is important in prognosis, and for stratifying and selecting patients for therapies targeting these facets of CD disease activity. Finally, cellular NOD2 protein levels may add further refinement to this assessment, since there is evidence for NOD2 expression in epithelial cells, whose level and function may contribute to mucosal-microbial homeostasis.[17]

Like NOD2, a number of additional genes or loci are promising for CD or UC diagnosis. The IBD2 (a chromosome 12 haplotype) and IBD5 (a 250-kb haplotype the 5q31 cytokine gene cluster) loci confer independent disease susceptibility with NOD2 in CD patients.[18,19] IBD2 is of additional interest because it is the strongest haplotype associated with UC disease risk, a form of IBD with historically less evidence for genetic susceptibility. This suggests that the pertinent gene will represent a common molecule regulating mucosal inflammation in both disease settings.[20] Even before the pertinent genes are identified, the IBD2 and IBD5 haplotypes are probably analytically suitable and merit assessment for their contribution to disease phenotype and prognosis.

Monitoring of certain candidate genes in innate immune function may find an eventual place in IBD clinical management, and we highlight four as examples of future prospects. First, the association of IBD with the major histocompatibility locus has been under investigation for decades.[21-23] While attention has previously focused on the HLA class 1 and 2 genes, the locus also includes TNF-α and lymphotoxin-α genes. A recent study by Taylor and colleagues indicates that certain haplotypes in this region predict responsiveness to anti-TNF (infliximab) therapy in CD.[24] This finding opens up the exciting possibility that such genotyping may be valuable in stratifying patients for biologicals or small molecules pertinent to the TNF facet of immune regulation or effector function. Second, troglitazone, a nominal agonist of PPAR-γ (or the closely related LXR family) have potent anti-inflammatory actions due to the regulatory action of these transcription factors on the macrophage activation state.[25,26] PPAR-γ epithelial expression is reported to be deficient in most UC patients, and also in some CD patients.[27] Since this class of small molecules is emerging as new therapy

in IBD (particularly CD), it would seem wise to assess the relationship and basis for impaired expression with the outcome of clinical response.

Third, defensins- a family of anti-microbial peptides produced by hemopoeitic and epithelial cells—have emerged as important players in epithelial-commensal bacterial interaction in the gut.[28] Fellerman and colleagues recently have reviewed evidence that impaired epithelial production of certain beta-defensins may contribute to disordered mucosal-microbiologic homeostasis in IBD, particularly UC.[29] Since changes in beta-defensin isoforms are heterogeneous, assessment of beta-defensin status may be a useful discriminator of biologic distinct patient subsets, whose prognosis or response to treatment would be more homogeneous.

Fourth, epithelial integrity forms an innate barrier to bacteria and their products, and impairments of this barrier result in chronic colitis in transgenic mouse systems.[30] P-glycoprotein (encoded by the multidrug resistance-1 gene, MDR1) is widely expressed in intestinal epithelial cells and forms a barrier to bacteria-dependent intestinal inflammation bacterial invasion and incursion of microbial products.[31] A recent study of a human MDR1 single nucleotide polymorphism C3435T, associated with lower intestinal P-glycoprotein expression, demonstrated that this polymorphism selectively predisposes to development of UC.[32] These observations suggest that assessment of polymorphic MDR1 expression, either in biopsy specimens or in blood lymphocytes may permit this trait to be integrated into a patient stratification approach.[33]

Anti-Microbial Antibodies and Adaptive Immune Components in IBD Diagnosis

Classic pathologic diagnostic parameters. The classic diagnostic parameters for UC and CD include pathologic and laboratory measurements, which in effect constitute applied cell biology and biochemistry. In the case of CD, this includes mucosal accumulation and activation of monocyte-macrophages and lymphocytes, with features of activation evocative of TH1-like responses, including granuloma formation (which experimentally is largely IFN-γ dependent).[34-36] Distinctly, UC is manifested not only by mononuclear cell infiltration, but also trafficking of granulocytes to form crypt abscesses, and cytokine and chemokine profiles mediating this effector response.[36-39] Disease activity is assessed in part by laboratory tests for inflammatory products of these immune-mediated processes. For example, serum acute phase reactant proteins (e.g., C-reactive protein) are elicited by the hepatocyte response to systemic levels of IL-1, IL-6, and TNF-α generated from the intestinal immune response.[25,40]

Anti-Microbial Antibodies

IgG seroreactivity to a variety of microbial species has been associated with IBD, particularly Crohn's disease.[41-43] These findings agree with the view that adaptive anti-microbial immunity is a component of CD pathogenesis, although the diversity of microorganisms raise several issues regarding the nature of the microbial encounter and immunologic response.[44] Nonetheless, such antibodies have emerged as useful analytes for IBD diagnosis.

The most widely used of these analytes is ASCA (antibodies to the cell wall polysaccharide of *Saccharomyces cerevisiae).* IgG and IgA antibodies are highly specific for CD, and moderately sensitive for this patient group (about 60% of patients).[45,46] The antigenic stimulus for these antibodies is uncertain, since the core epitope is found among diverse fungal and plant cell walls.[47] It is possible that the response relates to dietary antigens,[48,49] or a previously unappreciated existence of a fungal component in the enteric microbial community.[49] Finally, it is notable that studies of CD patients and first-degree family members indicate that ASCA seroreactivity (negative or positive) is a familial trait of both patients and unaffected family members.[50,51]

Table 1. Genetic loci in IBD disease stratification

Parameter	Description	Phenotype	Assay	Reference
IBD1	nod2	CD; fibrostenosing ileal disease	microsatellite	13-15
IBD2	12q14	nod2-independent CD; ulcerative colitis	microsatellite	18
IBD5	5q31	nod2-independent CD	microsatellite	19,20
MHC	LT-a	Infliximab response	microsatellite	24
β-defensins	Anti-microbial peptides	CD	Endoscopic biopsy immunohistochemistry	29
Mdr1	P-glycoprotein	UC	Single-nucleotide polymorphism; lymphocyte flow cytometry; sucrose absorption permeability	32,33
Thiopurine methlytransferase	Polymorphic enzyme controlling thiopurine bioavailability	6-mercaptopurine and azathioprine dosing	Erythrocyte enzyme activity	72

Mycobacterial species are associated with CD-like disease in human and other species, and several groups have reported elevated levels of anti-mycobacterial antibodies in CD patients.[52-56] Analytically, immunoassay detection has focused on recombinant protein antigens from *M. paratuberculosis* with impressive reports of specificity and sensitivity to CD (~75% and ~90%) compared to normals and UC patients.[57,58] While the contribution of *M. paratuberculosis* as a proinflammatory species in CD remains controversial, anti-mycobacterial antibiotics make the search for such a patient population appealing. Multiparameter assessment with serology and tissue based microbial detection may provide an avenue to identify such patients.[59]

TonB-linked outer membrane proteins of certain human intestinal commensals (*Bacteroides caccae, B. thetaiotamicron; E. coli*) have also emerged as useful antigenic targets for CD serodiagnosis.[60-62] These proteins (OmpW, SusC, and OmpC) are highly homologous to RagA of *Porphyromonas gingivalis*, an immunologic virulence factor for this periodontal disease pathogen. Similarly, the product of certain pseudomonads (the *P. fluorescens* protein PfiT, and the embedded I2 peptide) encode an antigen with ~60% IgG and IgA seroreactivity in CD patients.[62-64] Pseudomonads are rare commensals, and may be present in the gut lumen in part as a dietary component. However, *P. fluorescens* is molecularly detectable by PCR in the majority of CD lesions,[63] and PfiT itself has T cell superantigen bioactivity.[65] This implies that seropositive patients may display biologically distinct disease behavior useful in patient stratification (see below).[62]

IBD-Related T Cell Function

Disordered features of T cell microbial recognition and effector function anti-microbial T cell response is likely to be a central aspect of IBD disease biology.[66,67] To this point, we have lacked technologies for clinically useful assessment of the T cell in IBD. Minimal cells are

available from biopsy or resection specimens, and require elaborate handling unsuitable for the clinical setting; and, blood lymphocytes do not generally reflect the repertoire and differentiation state of tissue-based immune responses. An interesting solution may be now emerging from advances in the understanding of chemokine-based regulation of mucosal populations. CCR9, the receptor for a small-intestine specific chemokine TECK (CCL25), is highly expressed on blood lymphocytes recirculating to the small intestine.[68] Initial work indicates that this marker allows sampling of mucosal T cells "in transit", so they can be characterized with regard to antigenic specificities, activation state, or effector mechanisms reflective of the mucosal immune state. It appears that additional chemokine receptors may similarly permit assessment of other lymphocyte subpopulations and mucosal sites.[69]

T cell immunosuppression is a major strategy in IBD therapy, including the use of 6-mercaptopurine congeners acting through small G-protein inhibition of T cell activation.[70] An impediment to optimal clinical use of these agents is their substantial hematologic toxicity, which is not adequately predictable under conventional dosing. An important host parameter affecting bioavailability and metabolism is thiopurine methlytransferase, whose level of activity is genetically polymorphic.[71] A recent study suggested that measurement of this enzyme activity in surrogate cells (erythrocytes) can be useful in predicting exceptional resistance or toxicity with 6-MP and azathioprine.[72] This type of investigation highlights the opportunities for pharmacogenetic diagnostics as an early opportunity in facets of IBD clinical management.

Autoantibodies in IBD Diagnosis

Antibodies to a perinuclear neutrophil antigen, pANCA, are a sensitive and specific criterion for diagnosis of UC, and a clinically distinct subset of CD.[73,74] Antigenically, they are distinguished from other cANCAs and pANCAs by sensitivity of their antigen to DNAse I treatment and localization to the inner nuclear membrane leaflet.[75,76] At least a component of these antigens appear to include histone H1 and HMG family members.[77-80] It remains unknown whether these antibodies are a disease marker or a pathogenic factor. Recent advances in experimental autoimmune vasculitis have defined a comprehensive pathophysiologic mechanism relating cANCA (anti-MPO antibodies) to vascular and tissue damage.[81] This casts a fresh light on the previously reported occurrence of anti-endothelial antibodies in IBD,[82,83] and might serve as a guide to pathophysiologic assessment of pANCA in UC.

pANCA expression is a familial trait, since it is concordant in monozygotic twins, present in unaffected family members, and associated with a certain MHC II haplotype.[23,24,50,77,84] In genetic models of colitis in mouse, pANCA antibodies are also detected in UC-like phenotypes.[85] These observations indicate that pANCA expression is a trait associated with immunogenetic susceptibility for ulcerative colitis. Moreover, multiparameter assessment of this antibody trait and genetic polymorphisms (e.g., the TNFA/LTA locus) is useful not only in diagnosis, but in discrimination of a CD patient subset with a distinct distribution of disease and responsiveness to infliximab.[24,62,86,87]

Anti-epithelial antibodies, recently focusing on tropomyosin, have emerged as a disease-related seroreactivity in UC.[88-91] These antibodies are also observed in a number of mouse model systems preceding onset of clinical disease, and mediate antibody-dependent cytotoxicity in vivo.[85,92] Anti-tropomyosin antibodies thus would merit incorporation as part of a multi-parameter assessment (using genetic or other antibodies), to assess whether this trait would enhance current capabilities disease prognosis or management.

Multiparameter Analysis of Immunogenetic Traits in IBD Diagnosis

In this chapter, we have summarized different classes of immunogenetic parameters (genetic allelisms, and antibody levels to microbial and autoantigens) pertinent to IBD diagnosis, disease pattern, or response to treatment. Since these parameters are in most cases biologically divergent, it is reasonable to imagine that an integrated assessment of these traits in

Table 2. Serum antibodies in IBD disease stratification

Parameter	Description	Phenotype	Assay	Reference
ASCA	*Saccharomyces cerevisciae* cell wall polysaccharide	CD	ELISA	50,51
p35, IS900	*Mycobacterium paratuberculosis*	CD	ELISA; tissue PCR	59
OmpC OmpW	*E. coli,* *Bacteroides caccae*	CD	ELISA	60-62
PfiT (I2)	*Pseudomonas fluorescens*	CD	ELISA	62-64
CCR9 T cells	Homing receptor for small-intestine	CD disease activity	Flow cytometry	69
pANCA	Neutrophil perinuclear antigen	UC; infliximab-resistant CD	ELISA immunohistochemistry;	24,62,86,87
Tropomyosin	Anti-epithelial antibody	UC	ELISA	88-91
Antiendothelial		CD	Immunohistochemistry	82,83

patients would permit better resolution of clinically meaningful disease subsets. If so, such patient stratification could be useful for both IBD research, and to augment clinical assessment of patients for diagnosis and treatment.

Several studies have now provided evidence in support of this approach. As noted above, antibodies to certain microbial products and autoantigens (ASCA and pANCA) identify subsets of individuals with UC and CD with distinct clinica l courses.[50,87] Combined host genetic (MHC) and anti-microbial analysis have identified patient subsets distinguished by disease prognosis[23] or infliximab responsiveness.[24] Recently, our group has assessed a large CD patient population to assess their concordance with multiple anti-microbial antibodies (ASCA, PfiT, and outer membrane porins) and the pANCA autoantibody.[62] This study demonstrated several points about patient heterogeneity in CD. First, although each of the anti-microbial antibodies was present at similar frequencies in the CD population, the expression of these antibodies was nonconcordant, and in each individual was a stable phenotype. In particular, patients were divergent with respect to anti-fungal and anti-bacterial antibodies, indicating that these responses reflect different biologic groups of CD patients. Second, pANCA autoantibody levels were discordant with the two sets of anti-microbial antibody traits. Notably, these parameters were each stable over time in individual patients, suggesting that they are intrinsic traits of these patients. Taken together, this multiparameter analysis reflects the predicted biologic heterogeneity of the CD population (Fig. 1). Since NOD2 polymorphisms are already known to convey clinical heterogeneity distinct from some of these immunologic markers,[14] inclusion of NOD2 genotyping would be expected to further refine the definition of patients with regard to their clinical phenotype.

In summary, progress in basic IBD immunogenetics is now beginning to provide tools useful for clinically meaningful stratification of patients with this set of diseases. In some cases, available parameters may be useful as an adjunct to disease diagnosis. More important, these parameters, due to their reflection of distinct aspects of the disease biology, are likely to provide tools to identify patients with differing patterns of disease, prognosis, and response to treatment. Integrated multiparameter assessment is expected to be useful in stratification of more

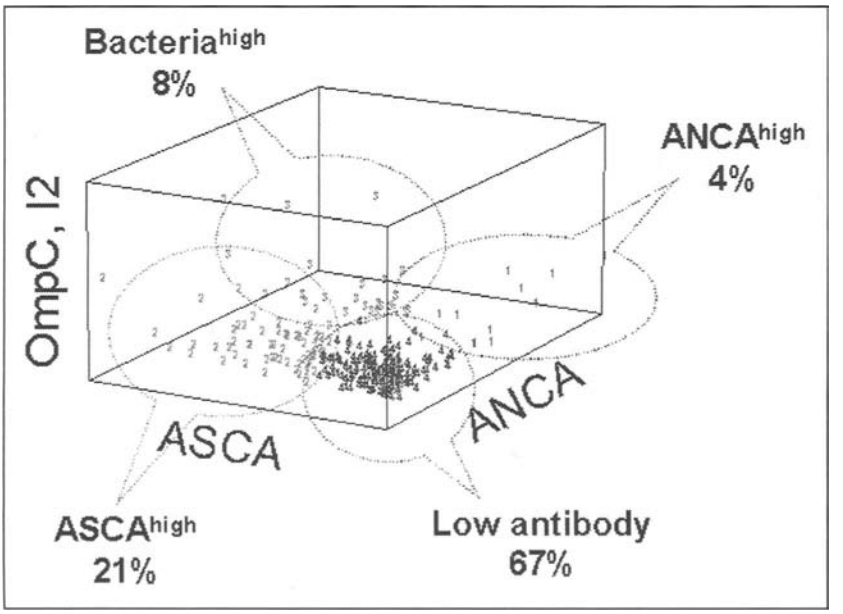

Figure 1. IBD patient stratification by multi parameter antibody analysis. A three-dimensional scatterplot was constructed from antibody levels using 5 specificities in 307 patients. Clusters of patients defined by antibody patterns are indicated by balloons.

biologically homogeneous patient subsets for functional and genetic pathogenesis research, trials of therapy, and guidance at the bedside in clinical management.

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