

THE mechanisms responsible for development of inflammatory bowel disease (IBD) have not been fully elucidated, although the main cause of disease pathology is attributed to up-regulated inflammatory processes. The aim of this study was to investigate frequencies of polymorphisms in genes encoding pro-inflammatory and anti-inflammatory markers in IBD patients and controls. We determined genotypes of patients with IBD ($n=172$) and healthy controls ($n=389$) for polymorphisms in genes encoding various cytokines (interleukin (IL)-1 β , IL-6, tumour necrosis factor (TNF), IL-10, IL-1 receptor antagonist). Association of these genotypes to disease incidence and pathophysiology was investigated. No strong association was found with occurrence of IBD. Variation was observed between the ulcerative colitis study group and the control population for the TNF- α -308 polymorphism ($p=0.0135$). There was also variation in the frequency of IL-6-174 and TNF- α -308 genotypes in the ulcerative colitis group compared with the Crohn's disease group ($p=0.01$). We concluded that polymorphisms in inflammatory genes are associated with variations in IBD phenotype and disease susceptibility. Whether the polymorphisms are directly involved in regulating cytokine production, and consequently pathophysiology of IBD, or serve merely as markers in linkage disequilibrium with susceptibility genes remains unclear.

Key words: Inflammatory bowel disease, Cytokine, Polymorphism

Inflammatory bowel disease: the role of inflammatory cytokine gene polymorphisms

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Introduction

Inflammatory bowel disease (IBD), a significant health problem in the developed world, comprises two chronic relapsing and remitting inflammatory disorders of the gastrointestinal tract: ulcerative colitis (UC) and Crohn's disease (CD).¹ UC, which has a slightly higher incidence than CD, affects only the large bowel, whereas CD can affect any part of the gastrointestinal tract. The general symptoms of UC are malaise, lethargy, and anorexia accompanied by diarrhoea with blood and mucus, abdominal discomfort, and fever.¹ The symptoms of CD tend to be more varied, depending on its site in the digestive tract, and can occur in a mild, moderate or severe form characterised by cycles of remission and active disease. These can include some or all of the symptoms of UC, although extra-intestinal manifestations are also common in CD such as inflammation of the eye, skin, joints, and mouth; arterial and venous thrombosis; chronic hepatitis; and other complications involving the liver, biliary tract, and kidneys.^{1–3}

Little is known about the underlying immunopathogenesis of IBD. However, both UC and CD are characterised by activation of macrophages and T

lymphocytes; pro-inflammatory cytokine, chemokine, and adhesion molecule expression; and an inability to adequately down-regulate immune activation. IBD, particularly CD, is characterised by a predominantly T helper (Th) 1 type profile, involving the up-regulation of cytokines such as interleukin (IL)-6, IL-8, IL-1 β , and tumour necrosis factor (TNF)- α .^{4–6} TNF antibody therapy has been found to improve both CD and UC, indicating a pivotal role for this cytokine in these conditions.^{7–9} Blocking IL-1 action with the IL-1 receptor antagonist (IL-1Ra) in animals with immune-complex-mediated colitis reduces the severity of inflammation and lowers eicosanoid concentrations in the bowel.^{10,11} Reduced levels of IL-10, an anti-inflammatory Th2 cytokine, are also potentially important in the pathogenesis of IBD, and encouraging results have been obtained with IL-10 therapy in IBD when administered topically in UC and systemically in active CD patients.^{12,13}

A genetic background resulting in up-regulated inflammation leading to IBD has been suggested by a number of studies, including that of Tysk *et al.* who estimated the heritability of CD (1.0, 95% confidence interval, 0.34–1.0) and UC (0.53, 95% confidence interval, 0.24–1.0) using the concordance rate in twin

pairs.¹⁴ Using 'age specific incidence data' to calculate the age-corrected empiric risk estimates, the risk of developing IBD has been determined to be 8.9% for offspring if either parent suffers IBD, 8.8% for siblings of a patient, and 3.5% for parents of an afflicted child.¹⁵

We postulated that small differences in cytokine levels as a result of gene polymorphisms may have an important effect on the inflammatory response and thus influence the pathophysiology of IBD. Here, we investigate frequencies of a range of polymorphisms in genes encoding pro-inflammatory cytokines (IL-1 β , IL-6, TNF- α , and TNF- β) and anti-inflammatory markers (IL-10, IL-1Ra) in a group of individuals with IBD and a large control population. We also aimed to compare genotype frequencies in UC and CD patients, in order to highlight possible differences between these two IBD disease groups. A number of the polymorphisms studied have been previously reported to have functional effects on the levels of the protein that they encode and have also been associated with various inflammatory and/or autoimmune diseases (Table 1).

Materials and methods

Patients and controls

Ethylenediamine tetraacetic acid anti-coagulated whole blood was collected from 389 healthy blood

donors (female, 42%; male, 58%; mean age, 37.1 years; age range, 18–65 years) and 172 patients with established IBD recruited from St James's Hospital, Dublin (female, 59%; male, 41%; mean age of disease onset, 30.8 years; median age of disease onset, 29.5 years; range of disease onset, 4–69 years). The disease group consisted of 64 patients with CD (female, 72%; male, 28%; mean age of disease onset, 30 years; median age of disease onset, 29 years; range of disease onset, 4–69 years) and 108 patients with UC (female, 52%; males, 48%; mean age of disease onset, 31.5 years; median age of disease onset, 30 years; range of disease onset, 12–61 years). Donor bloods for use as control samples were collected from The Northern Ireland Blood Transfusion Service ($n=60$) and The Blood Transfusion Service Board (Dublin and Cork and mobile units throughout the Republic of Ireland) ($n=329$). The study was approved by the St James's Hospital ethics committee and consent obtained from all participating subjects.

DNA isolation

DNA was extracted from whole blood using overnight proteinase K (1 mg/ml) cell lysis at 37°C in the presence of 0.5% sodium dodecyl sulphate followed by phenol/chloroform extraction and ethanol precipitation.

Table 1. Cytokine gene polymorphisms: effects on gene expression and disease associations

Gene	Polymorphism	Functional effect	Disease association*
IL-6	-174 G \rightarrow C ¹⁶	G: \uparrow IL-6 in normal individuals and in reporter gene assays (LPS/IL-1 stimulation) ¹⁶ No association with IL-6 levels in CAD patients ¹⁷ or in sepsis patients ¹⁸	S-JCA, ¹⁶ CAD ¹⁹
TNF- β	+252 A \rightarrow G (B2 \rightarrow B1) (TNFB) ²⁰	B2: \uparrow TNF- α expression in stimulated PBMC, ²¹ in severe sepsis patients ²² No effect on TNF- α ^{20,23} B1: \uparrow TNF- β in stimulated PBMC ²⁰ B2: \uparrow TNF- β in stimulated PBMC ²³ No effect on TNF- β in stimulated PBMC ²¹	Outcome in severe sepsis ^{22,24}
TNF- α	-308 G \rightarrow A	A: \uparrow TNF- α expression <i>in vitro</i> ²⁵ and <i>in vivo</i> ²⁶ No effect on TNF- α response to LPS ²⁷	Outcome in meningococcal disease, ²⁸ septic shock ²⁹
IL-10	-592 C \rightarrow A ³⁰	Generally no association	Mortality in critically ill patients ³¹
IL-10	-1082 G \rightarrow A ³⁰	A: \downarrow IL-10 expression in critically ill patients ³¹ G: \uparrow IL-10 expression in stimulated lymphocytes, ³⁰ in control and CD patients ³² No effect on IL-10 levels ²⁷	IBD ³³
IL-1RN	Intron 2, 86 bp VNTR ³⁴	A2: \uparrow IL-1Ra and \downarrow IL-1 α expression in GM-CSF-stimulated monocytes ³⁵ A2: \downarrow IL-1Ra in cultured PBMC ³⁶ A2: \uparrow IL-1 β in cultured PBMC ³⁷	IBD ³⁸
IL-1 β	+3953 C \rightarrow T ³⁹	T: \uparrow IL-1 β expression in LPS-stimulated monocytes ³⁹ No effect on <i>in vivo</i> IL-1 β levels ^{40,41}	IBD ⁴²

\uparrow or \downarrow , increase or decrease in expression compared with other alleles of the same polymorphism; \rightarrow , single base substitution; PBMC, peripheral blood mononuclear cells; CAD, coronary artery disease; S-JCA, systemic-onset juvenile chronic arthritis; VNTR, variable number of tandem repeats; LPS, lipopolysaccharide.

* Not all published disease associations are included in the table.

Polymerase chain reaction, restriction enzyme digestion, and agarose gel electrophoresis

Polymerase chain reaction (PCR) amplification of all polymorphic sites was performed in a 50 µl total volume. The standard reaction mix consisted of Taq DNA Polymerase buffer with MgCl₂ (Promega, Madison, WI, USA) (50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.1% Triton X-100, and 1.5 mM MgCl₂), 0.4 U of DNA Taq polymerase, 2 µl of genomic DNA, 4% dimethyl sulphoxide, 30 µM each of deoxyribonucleoside triphosphates, and 0.2 µM each of sense primer and antisense primer (Table 2). The cycling parameters for each assay are presented in Table 3, along with any changes to the standard PCR reaction mix. Restriction enzymes (New England BioLabs, Beverly, MA, USA; except *Hsp 92 II*, from Promega) used for each assay are presented in Table 2. The IL-6, TNF- α , IL-10-1082, and IL-10-592 PCR products were digested with the appropriate enzyme overnight at 37°C. The TNFB PCR product was digested for 3 h at 37°C, and the IL-1 β PCR product was digested for 12 h at 65°C. Restriction digest products were run in the appropriate percentage of agarose gel (Table 3) containing 1.6 µg/ml of ethidium bromide.

Statistical analysis

Allele and genotype frequencies were compared between patient and control groups, and between patient subpopulations, by means of the chi-squared test and Fischer's exact test when appropriate. A chi-squared $p < 0.05$ was considered statistically significant. Allele frequencies were determined by gene counting. Odds ratios and corresponding 95% confidence intervals were estimated by cross-tabulation. Statistics were performed using Statview software

(Statview version 4.5; Abacus Concepts Inc., Berkeley, CA, USA).

Results

IBD and controls

Genotype and allele frequencies for all seven polymorphisms in the control group, IBD group, UC group, and CD group are presented in Table 4. No association was found between any of the seven polymorphisms in this study and the occurrence of IBD, excepting the IL-1 β polymorphism where the CC genotype was at a higher frequency in the disease population compared with the control population (72% versus 61%, $p = 0.046$).

UC, CD and controls

The IBD study group can be divided into two groups: patients suffering from UC and those suffering from CD. A significant difference was observed when comparing the UC study group with the control population for the TNF- α -308 polymorphism, with carriers of the -308 A allele at a lower frequency in the UC group (28%) compared with the control group (40%) ($p = 0.02$). There was no association between any of the other polymorphisms studied and the occurrence of either form of IBD when comparing with the control group, although the IL-1RN (gene encoding the IL-1Ra protein) A2A2 genotype was almost twice the frequency in the CD group (14%) than that observed in either the control population (8%) or the UC population (7%) ($p = 0.088$). Our study found a significant difference in the frequency of IL-6-174 genotypes in the UC group (GG = 40%, GC = 41%, CC = 19%) compared with the CD group

Table 2. PCR primer sequences with the position of the 5' base, restriction enzymes, and cut sites

Primer	Sequence (5'-3')	Position of 5' base	Restriction enzyme	Restriction sites	
				Variant	Constant
IL-6-174 sense	ATGACTTCAGCTTTACTCTT	-324	Hsp 92 II	-174	-296
IL-6-174 antisense	ATAAATCTTTGTTGGAGGGT	-81			
TNFB sense*	CCCTCCTGCACCTGCTGCCTGG	+112	Hinf I	+251	+196
TNFB antisense*	AGAGGGG TGGATGCTTGGGTTC	+833			
TNF- α -308 sense	GGAGGCAATAGGTTTTGAGGGCCAT	-334	Nco I	-313	-162
TNF- α -308 antisense	CTGTCTCGGT TTCTTCTCCATGGCG	-140			
IL-10-1082 sense	TCTGAAGAAGTCCTGATGTC	-1248	Mnl I	-1085	-1196, -1191
IL-10-1082 antisense	CTCTTACCTATCCCTACTTCC	-1059		-1081	-1174
IL-10-592 sense	GACTCCAGCCACAGAAGCTTA	-967	Rsa I	-594	-884, -842,
IL-10-592 antisense	ATATCCTCAAAGTTCCCAAGC	-531			-834
IL-1 β +3953 sense	GTGTTGTATCAGACTTTGACCGTA	+3816	Taq I	+3952	+4052
IL-1 β +3953 antisense	GAGAGCTTTCAGTTCATATCGACCA	+4073			
IL-1RN sense**	CTCAGCAACTCCTAT	Intron 2	N/A	N/A	N/A
IL-1RN antisense	GCAGCAATAATGAAGAG	Intron 2			

* TNFB primers from Stüber *et al.*²²

** IL-1RN sense primer from Fang *et al.*²⁴

Table 3. PCR cycling parameters, changes to standard reaction mix, and percentage agarose gel used in each assay

Polymorphism	Cycles (n)	Denaturation	Annealing	Elongation	Changes to standard reaction mix	Agarose gel (%)
IL-6-174	40	94°C, 1 min	58°C, 1 min	72°C, 90 sec		3
TNFB*	37	95°C, 30 sec	68°C, 30 sec	74°C, 42 sec	1 µM each primer	2
TNF-α-308	40	94°C, 1 min	65°C, 1 min	72°C, 1 min		2
IL-10-1082	40	94°C, 1 min	58°C, 1 min	72°C, 1 min	No DMSO	4
IL-10-592	40	94°C, 1 min	64°C, 1 min	72°C, 1 min	1 µM each primer	3
IL-1β+3953	40	94°C, 1 min	65°C, 1 min	72°C, 1 min		3
IL-1RN 86 bp VNTR	40	94°C, 1 min	58°C, 1 min	72°C, 90 sec	1 µM each primer; 6% DMSO	2

DMSO, dimethyl sulphoxide; VNTR, variable number of tandem repeats.

* TNFB cycling parameters from Stüber *et al.*²²

(GG = 22%, GC = 64%, CC = 14%) ($p = 0.011$). A similar association was seen for the TNF-α-308 polymorphism (UC group, GG = 72%, GA = 21%, AA = 7%; CD group, GG = 53%, GA = 41%, AA = 1%; $p = 0.0135$).

Age of IBD onset

No association was seen between age of disease onset and any of the seven polymorphisms studied, either for the IBD group as a whole or when dividing into UC and CD groups.

Discussion

The pathophysiological mechanisms responsible for the development of IBD have not yet been fully determined. The main cause of disease pathology is,

as the name suggests, up-regulated inflammatory processes, the consequence of which is inflammatory cell infiltration in lamina propria, and crypt abscesses; goblet cell depletion in the case of UC; and inflammation in all bowel layers in CD, with lymphoid hyperplasia and an increased presence of chronic inflammatory cells.¹ Although infectious or other external agents are probable contributors to IBD pathogenesis, or might trigger disease onset, and the immune system clearly mediates tissue damage in the disease, it appears from available data that genetic factors play a role in determining the susceptibility of a given individual to IBD.¹⁵ Since IBD is characterised by a failure to confine the usual self-limited gut inflammatory response, genes involved in determining the level of the immune response in the inflammatory pathway might be risk factors in the disease. We therefore examined the potential association of a group of polymorphisms in cytokine

Table 4. Genotype counts (%) and allele frequencies of seven polymorphisms in a control population and an IBD patient population, and in subdivisions of the IBD population (UC and CD groups)

Polymorphism	Genotype	Genotype count (%)				Allele frequency				
		Control (n=389)	IBD (n=172)	UC (n=108)	CD (n=64)	Allele	Control	IBD	UC	CD
IL-6-174	GG	123 (32)	57 (33)	43 (40)	14 (22)	G	0.57	0.58	0.61	0.54
	GC	198 (51)	85 (49)	44 (41)	41 (64)	C	0.43	0.42	0.39	0.46
	CC	68 (17)	30 (18)	21 (19)	9 (14)					
TNFB	B1B1	52 (13)	25 (15)	18 (17)	7 (11)	B1	0.40	0.39	0.38	0.41
	B1B2	205 (53)	86 (50)	47 (43)	39 (61)	B2	0.60	0.61	0.62	0.59
	B2B2	132 (34)	61 (35)	43 (40)	18 (28)					
TNF-α-308	GG	233 (60)	115 (67)	78 (72)	37 (58)	G	0.78	0.81	0.83	0.78
	GA	140 (36)	49 (28)	23 (21)	26 (41)	A	0.22	0.19	0.17	0.22
	AA	16 (4)	8 (5)	7 (7)	1 (1)					
IL-10-1082	GG	123 (32)	61 (36)	37 (34)	24 (38)	G	0.55	0.58	0.56	0.60
	GA	180 (46)	77 (44)	48 (45)	29 (45)	A	0.45	0.42	0.44	0.40
	AA	86 (22)	34 (20)	23 (21)	11 (17)					
IL-10-592	CC	235 (60)	116 (68)	72 (67)	44 (69)	C	0.78	0.82	0.81	0.84
	CA	139 (36)	50 (29)	31 (29)	19 (30)	A	0.22	0.18	0.19	0.16
	AA	15 (4)	6 (3)	5 (4)	1 (1)					
IL-1β+3953	CC	240 (62)	124 (72)	78 (72)	46 (72)	C	0.78	0.83	0.83	0.83
	CT	125 (32)	38 (22)	24 (22)	14 (22)	T	0.22	0.17	0.17	0.17
	TT	24 (6)	10 (6)	6 (6)	4 (6)					
IL-1RN VNTR	A1A1	183 (47)	73 (43)	48 (44)	25 (39)	A1	0.69	0.66	0.685	0.617
	A1A2	159 (41)	70 (41)	44 (41)	26 (41)	A2	0.29	0.30	0.278	0.359
	A1A4	0 (0)	1 (0.5)	1 (1)	0 (0)	A3	0.02	0.03	0.028	0.024
	A2A2	31 (8)	18 (10)	8 (7)	10 (15)	A4	0.00	0.003	0.005	
	A1A3	15 (4)	9 (5)	6 (6)	3 (5)	A5	0.00	0.003	0.005	
	A1A5	0 (0)	1 (0.5)	1 (1)	0 (0)					
	A2A3	1 (0.2)	0 (0)	0 (0)	0 (0)					

VNTR, variable number of tandem repeats.

genes with the occurrence of IBD and of UC and CD patient subsets.

When comparing the complete IBD population with controls, a significant variation in genotype frequency of the IL-1 β promoter polymorphism was found. Higher levels of the pro-inflammatory cytokine IL-1 β would be expected to increase the likelihood of developing IBD, since higher levels of such cytokines occur in this disease. This study detected higher levels of IL-1 β CC in IBD patients, a genotype thought to be associated with low IL-1 β levels.³⁹ It is possible that the balance of high or low levels of IL-1 β is important in determining whether disease develops or in determining disease phenotype, rather than an overly pro-inflammatory genotype profile. It must also be noted that the association seen in this study was merely on the threshold of statistical significance ($p=0.046$) and thus needs further investigation in order to determine a real association with the disease.

In the study population, there was a significant variation in TNF- α -308 polymorphism genotype frequency when comparing the control group with the UC group. The control group contained a greater frequency of heterozygotes, and the UC group a greater frequency of homozygotes, especially GG, and thus the UC group had a lower frequency of carriers of the TNF- α -308 A allele. Previously, Vatay *et al.* found the TNF- α -308 A allele to be less frequent in both UC and CD patients compared with controls,⁴³ and Bouma *et al.* found a lower frequency of the TNF- α -308 A allele in UC patients compared with healthy controls.⁴⁴ However, Louis *et al.* found a modest non-statistically significant reduction of -308 A allele frequency in CD patients compared with both UC patients and controls.⁴⁵ Another study found no difference in TNF- α -308 allele frequencies between controls and patients with fistulising CD, but did not look at frequency in UC patients.⁴⁶ It therefore appears that there may be a slight association between the occurrence of UC and non-carriage of the -308 A allele. This allele is generally associated with increased serum TNF- α levels, and the significance of its non-association with UC, an inflammatory condition, is yet to be elucidated, although it is possible that this polymorphism might play a role in the differences seen between subsets of patients with UC and CD.

When comparing the two IBD disease groups, it was observed that there was a higher number of IL-6-174 and TNF- α -308 heterozygotes in the CD group compared with the UC group for both polymorphisms. TNFB genotypes also differed between UC and CD groups, but did not reach statistical significance; this difference was possibly caused by linkage of the TNFB polymorphism to the TNF- α -308 polymorphism. The explanation for why certain patient groups should contain a higher number of

heterozygotes or homozygotes is unclear. Rather than being directly associated with the disease, these genotypes may serve merely as genetic markers for other genes. The associations between IBD and the TNF- α polymorphism have already been discussed. The IL-6-174 polymorphism was previously investigated in 169 CD patients, 133 UC patients and 440 healthy controls, and no significant difference in allele, genotype, or carrier frequencies between patients and controls was observed.⁴⁷ The significance of the differences of the TNF- α and IL-6 polymorphisms between the UC and CD groups seen in our study is not clear, but points to a potential difference in the pathophysiology of these two diseases, and the possibility that the effect of higher or lower levels of TNF- α and IL-6 may play different roles in patients with UC and CD.

The IL-10-1082 polymorphism has been associated with susceptibility to IBD (UC or CD), and more significantly to UC alone;³³ however, our study found no evidence to support these results, concurring with the results of Klein *et al.* who looked at both -1082 and -592 polymorphisms in 142 CD patients, 104 UC patients and 400 controls, and found no differences in allele frequencies of either polymorphism between the groups studied.⁴⁸ Allele 2 of the IL-1RN gene has been found at a greater frequency in a UC population compared with a control group.³⁸ It is interesting to note that, although not statistically significant, the IL-1RN A2A2 genotype in our study was double the frequency in the CD group compared with both the UC and control groups, although this differs to the previous study where the association was for UC not CD. However, a previous study looked at the IL-1RN genotype in a large IBD population ($n=529$) (divided into IBD subgroups) compared with controls ($n=289$) and found no differences in genotype or allele frequencies, looking in particular at IL-1RN allele 2,⁴⁹ and it therefore appears likely that no real association between IBD and the IL-1RN VNTR exists. This lack of association has also been confirmed by a further study.⁵⁰

In conclusion, this study provides evidence that polymorphisms in genes involved in the inflammatory response participate in determining susceptibility and disease phenotype in IBD patients, although not to a highly significant degree. Whether the polymorphisms are directly involved in regulating cytokine production, and consequently disease pathophysiology of IBD, or serve merely as markers that are in linkage disequilibrium with susceptibility genes is unclear. There does not appear to be any clear-cut correlation between alleles or genotypes that are associated with higher pro-inflammatory cytokine levels and disease occurrence. However, it may be that an imbalance in levels of the cytokines is

an important factor in the development of IBD, rather than an overall pro-inflammatory profile.

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