INTERLEUKIN-10 PROMOTER POLYMORPHISMS IN RHEUMATOID ARTHRITIS AND FELTY’S SYNDROME


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SUMMARY

Objective. To examine whether promoter polymorphisms associated with variation in interleukin-10 (IL-10) production are relevant to the development of rheumatoid arthritis (RA) or Felty’s syndrome (FS).

Methods. DNA was obtained from 44 FS patients, 117 RA patients and 295 controls. The promoter region between −533 and −1120 was amplified by polymerase chain reaction, and polymorphisms detected by restriction enzyme digest or sequence-specific oligonucleotide probing.

Results. We found no significant difference in allele or haplotype frequencies between the groups.

Conclusion. There is no association between FS or RA and these recently identified IL-10 promoter polymorphisms. Other genetic or environmental factors could explain the alterations in IL-10 levels seen in these conditions.

Key words: IL-10, Polymorphism, Rheumatoid arthritis.

INTERLEUKIN-10 (IL-10) was originally described as cytokine synthesis inhibitory factor, because of its ability to inhibit the production of IL-2, IL-3, interferon gamma and granulocyte-macrophage colony stimulating factor (GM-CSF) by Th 1 clones [1]. It is produced by T cells, B cells, macrophages/monocytes and keratinocytes, has immunoregulatory effects by downregulating class II major histocompatibility complex (MHC) expression, and inhibits the production of pro-inflammatory cytokines by monocytes [2]. Conversely, it has stimulatory effects on B cells, including an increase in expression of MHC class II [3] and production of immunoglobulin, and DNA replication [4]. IL-10 secretion appears, therefore, to be important in immune regulation, contributing to the control of the balance between humoral and inflammatory responses.

There are several pieces of evidence to suggest that IL-10 may be important specifically in rheumatoid arthritis (RA). Although one study has shown reduced IL-10 in RA blood compared with normal individuals [5], another by Cush et al. [6] demonstrated increased blood levels both by ELISA and semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR). Both groups demonstrated elevated IL-10 levels in RA synovial fluid. More recently, monocytcs from the peripheral blood of RA patients have been shown, by RT-PCR, to produce significantly more IL-10 spontaneously than those from normal individuals [7]. Others have studied clones derived from RA synovium, demonstrating increased production compared with clones from the blood [8, 9]. Lately, it has been shown that cultured synovial fluid mononuclear cells (SFMC) derived from RA patients produce IL-10 spontaneously [10]. Furthermore, using antibody blocking experiments, endogenously produced IL-10 was shown to inhibit production of IL-1β, tumour necrosis factor alpha (TNF-α) and GM-CSF by SFMC. Using an in vitro model to study the effects of RA mononuclear cells (MNC) on cartilage degradation, van Roon et al. [11] showed that IL-10 could reverse the degradation induced by antigen-stimulated MNC. Moreover, IL-10 had a stimulatory effect on proteoglycan synthesis. Taken together, these studies suggest an important role for IL-10 in the pathogenesis of RA.

Felty’s syndrome (FS) is a rare complication of RA characterized by neutropenia and often splenomegaly. Compared with age-matched RA patients, FS sufferers have less joint inflammation and high levels of rheumatoid factor positivity [12]. IL-10 production has not been measured in FS, but it can be argued that as a patient progresses from RA to FS, a switch from predominantly cell-mediated to humoral immunity occurs. Hence, IL-10 may have an important role in changing the disease phenotype.

Three polymorphisms within the promoter of the IL-10 gene have recently been identified, their gene frequencies studied in a healthy population, and their effect on production of the cytokine by lymphocytes in vitro assessed [13]. One of the polymorphisms, at position −1087 from the transcription start site, was associated with varying levels of cytokine production after stimulation with concanavalin A (Con A). This was compatible with an earlier study using the luciferase assay to show that a positive regulatory region resided between −1100 and −900 [14]. There are clearly many reasons why IL-10 production might be elevated in RA or FS, but one possibility is that a genetic predisposition to high IL-10 production might...
be an important contributory factor. Indeed, recent work has demonstrated a high degree of heritability of IL-10 production from lymphocytes stimulated in vitro [15]. For this reason, we chose to study the frequencies of promoter polymorphisms in two well-characterized patient populations.

PATIENTS AND METHODS

Patients

In Manchester, 117 RA patients were studied, all of whom fulfilled the 1987 revised criteria of the American College of Rheumatology (ACR). At Guy’s, 44 patients with FS were studied. These have been well characterized immunogenetically for HLA class II gene polymorphisms, and for T-cell phenotype and clonality [12]. All these fulfilled the ACR criteria for RA, and in addition had unexplained neutropenia of >6 months duration. Splenomegaly was not considered an essential criterion.

Controls

For the Manchester controls, 119 kidney donors from north-west England were used for frequency analysis. The London controls for genotyping came from panels of DNA from normal healthy volunteers maintained in the tissue typing laboratories at Guy’s and King’s College Hospitals.

DNA extraction

DNA was extracted either from fresh whole blood or from lymphocytes previously frozen in liquid nitrogen, by proteinase K digestion, phenol and chloroform extraction. DNA was then precipitated with ethanol.

DNA amplification

PCR amplification was performed on a Perkin Elmer 9600 thermal cycler in 30 µl reaction mixtures containing test DNA, 50 mM KCl, 10 mM Tris–HCl, 0.1% Triton X-100, 2.5 mM MgCl₂, 200 µM each dATP, dGTP, dTTP and dCTP (Gibco BRL), 0.5 µM each primer, 0.4 mM betaine (Sigma) and 1 U Taq polymerase (Gibco BRL). The following cycling conditions were used: 95°C 3 min; 30 cycles of 95°C 30 s, 56°C 30 s, 72°C 1 min; 72°C 3 min. Primers were used which amplified the segment of promoter from −1120 to −533; upstream 5'-ATCCAAGACAAACTACTAA-3'; downstream 5'-TAAAATTCCTCAAGAGGTTCC-3'.

Restriction enzyme genotyping

For the FS and control patients studied in London, genotyping of IL-10 promoter polymorphism was modified from a previous method [13], using restriction fragment length polymorphism (RFLP). An 8 µl aliquot of PCR product was digested overnight with 1 µl of MaeIII (Pharmacia Biotech) for the −824 polymorphism, or Rsal (New England Biolabs) for the −597 polymorphism, and separated by size on a 2 or 3% low-melting-point agarose gel, respectively. Each gel was stained with ethidium bromide and visualized under UV light.

Sequence-specific oligonucleotide probing (SSOP)

RA patients and controls from Manchester were studied for all three polymorphisms by SSOP, as described [13]. For the London group, only the −1087 polymorphism was studied by this method. Two oligonucleotide probes were used to detect each allele of the −1087 polymorphism by a dot blot technique. Ten microlitres of PCR product were blotted onto Hybond N + nylon transfer membrane (Amersham) and probes were hybridized at 50°C.

The probe for IL-10 1087*G (TTCTTTGGGAG GGGGAAG) was washed stringently at 53°C, and that for IL-10 1087*A (ACTTCCCTTCCCAA AGAA) at 55°C. Probes and conditions for the −824 and −597 polymorphisms were as previously described [13].

Polymorphism investigation

There are three polymorphisms of the IL-10 promoter which have been described previously [13]. The first at −1087 constitutes a G to A substitution, the second at −824 is a C to T substitution, and the third at −597 is a C to A substitution. The −824 substitution leads to the destruction of an MaeIII restriction site, hence the −824 C allele gives rise to fragments at 79, 217 and 292 bp, and the −824 T allele has bands at 79 and 509 bp. The −597 substitution introduces an Rsal restriction site, so that the −597 C allele gives rise to fragments of lengths 42, 232 and 306 bp, compared with 42, 66, 232 and 240 bp for the −597A allele. Heterozygous individuals were identified by the presence of an additional band.

Statistical analysis

Differences between control and patient allele or haplotype frequencies were measured by the χ² test, with Yates’ correction where appropriate.

RESULTS

Polymorphism frequency

Since different methods were used to study polymorphism between the two centres, it was important to ensure consistency of results. DNA samples from Manchester of known genotype were therefore analysed in London, and the same results obtained. Hence, there was agreement between the SSOP and RFLP methods.

Figures 1a and b show typical results of genotyping for the −824 and −597 polymorphisms by RFLP. Figure 1c shows representative results of SSOP genotyping for the −1087 polymorphism. The various alleles can clearly be distinguished.

Allele and haplotype frequencies are summarized in Tables I and II. Allele frequencies were calculated for the Manchester and London control panels independently. Both were in Hardy–Weinberg equilibrium, with identical allele frequencies. The two groups were therefore combined for statistical analysis. Three putative haplotypes were formed: GCC, ACC and ATA for the −1087, −824 and −597 polymorphisms, respectively. No significant difference in allele or haplotype
Fig. 1.—(a) Typical results of the MaeIII digest in respect of the polymorphism at position −824. Individuals 1, 3, 5, 7, 9 and 10 are homozygous for −824*C; individual 2 is homozygous for −824*T; 4, 6 and 8 are heterozygous. The last lane shows a 50 bp ladder. Representative results for the RsaI digest of the polymorphism at position −597. Individuals 4 and 5 are homozygous for −597*C; subject 3 is homozygous for −597*A; 1 and 2 are heterozygous. The last lane shows a 50 bp ladder. Note that these illustrations were prepared independently of each other. Hence, individual 1 in (a) is not the same person as individual 1 in (b) or (c). Representative results of the hybridization with probes for the polymorphism at position −1087. Individuals 1, 5, 6, 9 and 11 are homozygous for −1087*G; 2 and 7 are homozygous for −1087*A; 3, 4, 8 and 10 are heterozygous.

frequencies was seen between controls, RA and FS patients. The numbers studied allowed an 80% power to detect an odds ratio of 1.3 for RA, and an 80% power to detect an odds ratio of 1.5 for FS, for the most common haplotype.

**DISCUSSION**

This study of patients with RA and FS demonstrated no difference in the frequency of IL-10 promoter polymorphisms between RA and FS patients and controls. It was sufficiently powerful to ensure a low probability of missing a biologically relevant

**TABLE I**

Allele frequencies. There is no significant difference in allele frequencies between the three groups.

<table>
<thead>
<tr>
<th>Allele</th>
<th>Control (n = 295)</th>
<th>RA (n = 117)</th>
<th>Felty (n = 44)</th>
</tr>
</thead>
<tbody>
<tr>
<td>−1087*G</td>
<td>0.51</td>
<td>0.5</td>
<td>0.54</td>
</tr>
<tr>
<td>−1087*A</td>
<td>0.49</td>
<td>0.5</td>
<td>0.46</td>
</tr>
<tr>
<td>−824*C</td>
<td>0.79</td>
<td>0.78</td>
<td>0.83</td>
</tr>
<tr>
<td>−824*T</td>
<td>0.21</td>
<td>0.22</td>
<td>0.17</td>
</tr>
<tr>
<td>−597*C</td>
<td>0.79</td>
<td>0.78</td>
<td>0.83</td>
</tr>
<tr>
<td>−597*A</td>
<td>0.21</td>
<td>0.22</td>
<td>0.17</td>
</tr>
</tbody>
</table>

**TABLE II**

Haplotype frequencies. Three putative haplotypes are formed as above, termed H1, H2 and H3. There is no significant difference in the haplotype frequencies.

<table>
<thead>
<tr>
<th>Haplotype</th>
<th>Control (n = 295)</th>
<th>RA (n = 117)</th>
<th>Felty (n = 44)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H1 (G-C-C)</td>
<td>0.5</td>
<td>0.5</td>
<td>0.53</td>
</tr>
<tr>
<td>H2 (A-C-C)</td>
<td>0.28</td>
<td>0.28</td>
<td>0.31</td>
</tr>
<tr>
<td>H3 (A-T-A)</td>
<td>0.22</td>
<td>0.22</td>
<td>0.16</td>
</tr>
</tbody>
</table>
contribution of the polymorphisms to disease susceptibility. Although the polymorphisms were not associated with RA, our data do not exclude the possibility of an association with subsets of RA other than FS. There is clear evidence to support an important role for IL-10 in the pathogenesis of RA [6]. The cytokine has well-described anti-inflammatory effects which may be employed in an attempt to downregulate an inappropriate immune response. However, the results of this investigation suggest that factors other than variation in the gene promoter are responsible for the elevated levels seen within the joint, and arguably the blood, of RA patients. This contrasts with findings in systemic lupus erythematosus, another autoimmune condition with elevated IL-10 levels, where significant associations have been found with a microsatellite polymorphism in the IL-10 promoter region [16].

RA is a complex disease, with many elements of the immune system activated. In this context, was it unreasonable to hypothesize that elevated levels of one cytokine might arise from single-base-pair polymorphisms in the gene promoter? A recent study by Westendorp et al. [15] examined the heritability of cytokine levels by stimulating lymphocytes ex vivo from first-degree relatives of patients with meningococcal disease and healthy monozygotic twins. It showed that 75% of the variation in IL-10 production was genetically determined. Furthermore, although TNF-α production was also under strong genetic control (65%), there was no association between the polymorphisms at −308 and −238 in the gene promoter and protein production ex vivo. Hence, this study demonstrates the high degree of heritability of IL-10 production, and raises the possibility, by analogy with the TNF-α findings, that there may be genetic factors outside the gene promoter which underlie the elevated IL-10 levels seen in RA patients. Further work will be necessary before any such factors can be identified, and their relationship to RA determined.

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References