No evidence for involvement of the Toll-like receptor 4 (TLR4) A896G and CD14-C260T polymorphisms in susceptibility to ankylosing spondylitis

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EXTENDED REPORT

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OBJECTIVES: Ankylosing spondylitis (AS) is a multifactorial and polygenic disease. Apart from HLA, other genes very probably play a role in disease susceptibility. Indigenous bacteria of the gastrointestinal flora appear to play a role in the pathogenesis of the disease; therefore, genes controlling the innate and acquired immune response are good candidates to study disease susceptibility. CD14 and Toll-like receptor 4 (TLR4) are key receptors for the sensing of bacteria. The CD14 C-260T and TLR4 A896G single nucleotide polymorphisms are associated with aberrant signal transduction for bacterial agonists.

METHODS: The distribution of the CD14 C-260T and TLR4 A896G polymorphisms was studied in genomic DNA from 113 unrelated white Dutch AS patients and 170 ethnically matched control subjects. The diagnosis of AS was made according to the modified New York criteria. The CD14 C-260T and TLR4 A896G polymorphisms were genotyped by PCR-RFLP methods.

RESULTS: No significant differences were found between patients and controls in the frequencies of the CD14 C-260T allele (odds ratio 0.65; 95% confidence interval 0.37 to 1.15) or the TLR4 A896G allele (1.68; 0.67 to 4.19).

CONCLUSIONS: There is no evidence for involvement of the CD14 C-260T or TLR4 A896G polymorphisms in susceptibility to AS. An important role of bacteria and genetic predisposition of the innate immune system in cases of AS cannot be excluded by these findings. Therefore, studies of the surprisingly highly polymorphic candidate genes in this field should be continued.

Despite the discovery of the association between ankylosing spondylitis (AS) and HLA-B27 over thirty years ago, the precise role of the adaptive and innate immune system in the pathogenesis of this common rheumatic disorder has not been clarified.

Apart from genetic factors, bacteria are thought to be crucial in the pathogenesis of AS, the prototype of the spondyloarthropathies. Over the past decades it has been shown that bacterial infections can trigger the onset of at least one type of spondyloarthropathy, reactive arthritis (ReA). ReA occurs frequently after infectious diarrhoea caused by Shigella flexneri, Salmonella typhimurium and enteritides, Yersinia enterocolitica, or Campylobacter jejuni, or can be caused by urogenital infections with Chlamydia trachomatis (for a review, see Zeidler and Schumacher). However, so far no specific pathogen has been consistently linked to the development of AS.

The acquired and the innate immune systems play important roles in the host defence against pathogens. Innate immunity refers to the inborn resistance that is already present the first time a pathogen is encountered; it does not require prior exposure and is not modified significantly by repeated exposures to the pathogen over the life of an individual. Acquired immunity refers to resistance that is weak or absent on first exposure, but that increases dramatically with subsequent exposures to the same specific pathogen.

Cluster of differentiation 14 (CD14) and Toll-like receptor 4 (TLR4) are, together with the MD-2 protein, part of the lipopolysaccharide (LPS) receptor complex of the innate immune system. LPS is a glycolipid specific to gram negative bacterial cell walls. CD14 is the receptor of monocytes/macrophages for complexes of LPS and LPS binding protein (LBP). LPS is transferred from a CD14/LBP complex to a TLR4/MD-2 membrane receptor complex at the cell surface. A transmembrane signal leads to nuclear factor (NF)-κB activation, subsequent induction of gene transcription and release of pro-inflammatory cytokines, such as tumour necrosis factor α, initiating inflammation.

Besides LPS, other bacterial and host agonists are reported to be recognised by TLR4 including C. trachomatis and host heat shock protein 60 (HSP60) ligands.

Although, to the best of our knowledge there is no evidence for linkage in the region of the CD14-260 single nucleotide polymorphism (SNP) on chromosome 5q31, functional studies imply this gene may be of relevance to AS. The CD14-260 promoter SNP enhances transcriptional activity, and is associated with enhanced monocyte CD14 expression.

Individuals carrying the TT genotype have significantly higher serum levels of soluble CD14 than have those with CC or CT genotypes and they have increased density of CD14 in blood monocytes.

Recently, allele CD14-260T has been found to be significantly positively associated with ulcerative colitis (UC) in Japan. However, in Germany, the allele CD14-260T and the genotype TT were increased in Crohn’s disease (CD) but not in UC. More recently, the same group of investigators found allele CD14-260T and TT genotype frequencies of the CD14 SNP to be significantly increased only in patients with CD carrying at least one of the susceptibility variants Arg702Trp, Ser716Leu and Asp299Glu.

ABBREVIATIONS: AS, ankylosing spondylitis; CD, Crohn’s disease; HSP, heat shock protein; LBP, lipopolysaccharide binding protein; LPS, lipopolysaccharide; NF, nuclear factor; SNP, single nucleotide polymorphism; TLR, Toll-like receptor; UC, ulcerative colitis
Gly908Arg, or Leu1007fsinsC in the CARD15 gene. Both UC and CD can have disease manifestations that belong to the spondylarthropathies.

So far, there have been no studies addressing the possible association of the CD14-C260T SNP with AS, although carriage of the CD14-260T allele might increase the susceptibility of female patients to the development of chronic spondylarthropathy.

The TLR4 gene is located on chromosome 9q32-q33 at 115.8 Mb. Genome scanning in AS identified linkage to a region on chromosome 9q. Marker D9S1826 on chromosome 9q34 at 133.9 Mb achieved a lod score of 2.8 (p<0.0005), and marker D9S1682 at 120.4 Mb achieved a lod score of 2.3 (p<0.0005), both suggestive of linkage.17 Given the distance between the TLR4 gene and the D9S1826 and D9S1682 markers, it is unlikely that TLR4 can warrant the linkage to the region on chromosome 9. Nevertheless, similar to the situation in CD14, functional studies imply that TLR4 might be of relevance to AS.

The A896G SNP is located in the ectoplasmic receptor domain of TLR4, resulting in an aspartic acid to glycine substitution at position 299. Recently, the A896G SNP was shown to be associated with an "endotoxin hyporesponsive phenotype" in concert with other genetic changes or acquired factors that influence the complex physiological response to LPS.18 However, monocytes from individuals heterozygous for allele T896G do not exhibit a deficit in recognition of LPS from several bacterial strains.19 Moreover, the heterozygous TLR4 A896G polymorphism does not influence LPS induced human whole blood cytokine release.20 Other agonists for TLR4 have not yet been tested functionally.

The present study was designed to study the frequency of the CD14 C-260T and TLR4 A896G SNPs in AS to find out whether these polymorphisms contribute to disease susceptibility and clinical manifestations.

PATIENTS AND METHODS

Subjects
After informed consent, 113 AS patients were recruited from the outpatient Department of Rheumatology of the Jan van Breemen Institute. All AS patients fulfilled the diagnosis of AS, according to the modified New York criteria.21 Controls were 170 randomly selected healthy blood donors from the Amsterdam region. All subjects were unrelated Dutch whites.

Methods
Genotyping the CD14 C-260T SNP (NCBI SNP cluster ID: rs2569190) was performed with forward primer 5'-TGACCTTGCCCACTTGCTCTTT-3' and 5'-CCTGCGAGAATGCCGTCTT-3'. Digestion overnight with HaeIII (New England Biolabs, UK) of the 107 bp amplicons was followed by electrophoresis on 4% agarose gels, staining with ethidium bromide, and visualisation under ultraviolet light, and resulted in two fragments of 83 and 24 bp (C allele) or 107 bp (T allele).

Genotyping of the TLR4 A896G SNP (NCBI SNP cluster ID: rs4986790) was performed as described by Morre et al.22

Statistical analysis
Allele and genotype frequencies were tested for Hardy-Weinberg equilibrium by χ² test. To compare frequencies, χ² or Fisher’s exact test was used. The possible influence of the two studied polymorphisms in patients and controls was analysed by logistic regression. A two sided p value <0.05 was considered significant. The magnitude of association was expressed as odds ratio with a 95% confidence interval (CI). Statistical analysis was performed by SPSS 10.0 for Windows.

RESULTS
Characteristics of the 113 AS patients are summarised in table 1. Genotype and allele frequencies in AS patients and controls for the CD14 C-260T and the TLR4 A896G SNP in table 2. The genotype frequencies in the control group did not deviate from HWE equilibrium. No significant differences were observed between AS patients and controls in the frequencies of carrierrship of the CD14-260T and TLR4 A896G alleles (table 2). Logistic regression analysis showed absence of modification of the odds ratios (OR). Thus, no interaction between the CD14 C-260T and TLR4 A896G polymorphisms was found (data not shown).

No significant associations were found between carriage of each of the alleles CD14-260T and TLR4 A896G with sex, existence in past or present of peripheral arthritis or acute anterior uveitis, age at first complaints, years between first complaints and actual diagnosis of AS, or the number of patients with at least one first degree family member with AS (table 3).

DISCUSSION
The studied SNPs reported to affect gene function in two genes that play a role in innate immunity, CD14 C-260T and TLR4 A896G, are not significantly associated with the susceptibility to or the clinical manifestations of AS.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Demographic and clinical characteristics of patients with ankylosing spondylitis (n = 113)</th>
</tr>
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<tbody>
<tr>
<td></td>
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<tr>
<td>Characteristics</td>
<td></td>
</tr>
<tr>
<td>Age, years*</td>
<td>52.6 (12.5; 19 to 79)</td>
</tr>
<tr>
<td>Age of first complaints, years†</td>
<td>23.0 (19.0 to 31.5)</td>
</tr>
<tr>
<td>Age of actual diagnosis, years†</td>
<td>34.0 (25.0 to 42.5)</td>
</tr>
<tr>
<td>Years between first complaints and diagnosis†</td>
<td>7.0 (2.0 to 12.5)</td>
</tr>
<tr>
<td>Women</td>
<td>12.5%</td>
</tr>
<tr>
<td>HLA-B27 positive</td>
<td>98%</td>
</tr>
<tr>
<td>Indocylloids</td>
<td>40%</td>
</tr>
<tr>
<td>Peripheral arthritis</td>
<td>37%</td>
</tr>
<tr>
<td>First degree relatives with AS</td>
<td>28%</td>
</tr>
<tr>
<td>*Mean (SD; range), †median (interquartile range).</td>
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</tbody>
</table>

<table>
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<tr>
<th>Table 2</th>
<th>Allele and genotype frequencies of the CD14 C-260T and the TLR4 A896G polymorphisms in ankylosing spondylitis patients and controls</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>CD14 C-260T</td>
<td></td>
</tr>
<tr>
<td>Allele</td>
<td>(n = 113)</td>
</tr>
<tr>
<td>C</td>
<td>111 (49.1%)</td>
</tr>
<tr>
<td>T</td>
<td>115 (50.9%)</td>
</tr>
<tr>
<td>Genotype</td>
<td></td>
</tr>
<tr>
<td>CC</td>
<td>23 (20.4%)</td>
</tr>
<tr>
<td>CT</td>
<td>65 (57.5%)</td>
</tr>
<tr>
<td>TT</td>
<td>25 (22.1%)</td>
</tr>
<tr>
<td>TLR4 A896G</td>
<td></td>
</tr>
<tr>
<td>Allele</td>
<td>(n = 113)</td>
</tr>
<tr>
<td>A</td>
<td>219 (96.9%)</td>
</tr>
<tr>
<td>G</td>
<td>7 (3.1%)</td>
</tr>
<tr>
<td>Genotype</td>
<td></td>
</tr>
<tr>
<td>AA</td>
<td>106 (93.8%)</td>
</tr>
<tr>
<td>AG</td>
<td>7 (6.2%)</td>
</tr>
<tr>
<td>GG</td>
<td>0 (0%)</td>
</tr>
</tbody>
</table>

No significant difference was found between patients and controls in the frequencies of the carrierrship of the CD14-260T (OR 0.65; 95% CI 0.37 to 1.15) or TLR4 A896G (OR 1.68; 95% CI 0.67 to 4.19) allele.
Nevertheless, this study cannot exclude that specific phenotypic features of AS are related to TLR4 and/or CD14 genotypes. Therefore, it would be of interest to assess whether the CD14 C-260T and TLR4 A896G SNPs are associated with disease severity (C-reactive protein, ESR, Bath Ankylosing Spondylitis Disease Activity Index, Bath Ankylosing Spondylitis Functional Index), anatomical evolution (Bath Ankylosing Spondylitis Metrology Index, sacroiliitis, degree of ankylosis), or involvement of the gut. Moreover, although this study could not find a significant association between CD14 C-260T and TLR4 A896G and the clinical manifestations of AS, the number of patients in this analysis was too low to warrant a definitive conclusion.

Bacteria are thought to play a crucial role in the pathogenesis of AS. In animal models such as B27 transgenic rats, in ~50% of the cases, the presence of the bacterial flora is obligatory for development of inflammatory gut lesions and peripheral and axial inflammatory joint lesions, similar to AS. When raised in a germfree environment, inflammatory and peripheral and axial inflammatory joint lesions, similar to AS occurs frequently in patients with newly diagnosed inflammatory bowel disease—that is, UC or CD. On the other hand, patients with spondylarthropathies often have subclinical gut involvement. This gut inflammation is clinically and histologically closely related to CD.

It is likely that an altered host response of the innate immune system may contribute to the development of AS. This hypothesis is supported by the findings on genetic susceptibility of CD, which have shifted the focus of research in this disease towards innate immunity. The relationship with acquired immunity provides a good insight into the mechanisms that control inflammation in the gut. Recently, IgA levels of anti-Saccharomyces cerevisiae mannan antibodies, a marker of CD, have been found to be significantly higher in patients with spondylarthropathies, and more specifically in AS, than in healthy controls and patients with rheumatoid arthritis.

Besides the association reported with CD14, an established genetic association of the innate immune system with CD is the Leu1007IleSNp insertion mutation at position 3020 in the CARD15 gene, located on chromosome 16p12. This mutation was described as an important susceptibility factor for CD. However, studies from our group in the Netherlands, and from Spain and the UK have disproved

<table>
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<tr>
<th>Characteristics</th>
<th>TLR4 896G</th>
<th>CD14 260T</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Carrier</td>
<td>Non-carrier</td>
</tr>
<tr>
<td>Female/male</td>
<td>1/6</td>
<td>12/85</td>
</tr>
<tr>
<td>PA (+/−)</td>
<td>3/4</td>
<td>35/60</td>
</tr>
<tr>
<td>AAU(+/−)</td>
<td>5/2</td>
<td>34/57</td>
</tr>
<tr>
<td>Familial(+/−)</td>
<td>2/4</td>
<td>23/61</td>
</tr>
<tr>
<td>Age of first complaints, mean (SD) (range)</td>
<td>23.83 (6.18)</td>
<td>25.64 (9.78)</td>
</tr>
<tr>
<td>Years to actual diagnosis, mean (SD) (range)</td>
<td>9.08 (11.43)</td>
<td>9.27 (8.83)</td>
</tr>
</tbody>
</table>

AAU, acute anterior uveitis; PA, peripheral arthritis; familial, patients with at least one first degree family member with AS; +/−, present/not present; years to actual diagnosis, number of years between the first complaints and the actual diagnosis of AS. *Mann-Whitney test

The identification and functional characterisation of mutants, such as those in CD14, TLR, and NOD2/CARD15 receptors, provide a new insight into the relationships between bacteria and the host, and between bacteria and the development of disease. A previous study in Germany in a population of similar ethnic background to the one we have studied has revealed interactions of the CARD15 and CD14 genes that increase the risk for developing CD. Interestingly, so far the associations found in the three genes CD14, CARD15, and TLR4 have not been reproduced in all population studies demonstrating the complexity of the interaction between genes and environment. As recently described by Vercelli, ‘Genetic variation in innate immunity genes at the interface with the environment may skew the human immune response in critical ways by modulating the impact of pathogen exposure’. Therefore, although we found no significant associations between the CD14-C260T and TLR4 A896G SNPs with AS in general or with clinical characteristics, the significance of functional polymorphisms in genes that are involved in innate immunity deserve to be studied in this disease, in which bacteria and HLA appear to be important in the pathogenesis.

These are early days in the studies of innate immunity in AS and in many other chronic inflammatory diseases. It is possible that polymorphisms in other receptors sensing other bacteria than the ones recognised by CD14, TLR4, and CARD15 may prove to be important in understanding the synergy between clinical manifestations, epidemiology, molecular biology, and pathophysiology of inflammation.

ACKNOWLEDGEMENTS

The authors are grateful to J Pleijster for assistance in genotyping the CD14 C-260T and TLR4 A896G polymorphisms.

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A S Peña, the Department of Gastroenterology
B A C Dijkmans, J E van der Horst-Bruinsema, and the Department of Rheumatology, VU University Medical Centre, Amsterdam, the Netherlands.
Lipopolysaccharide is in close proximity to each of the proteins in its complexes of lipopolysaccharide (LPS) and LPS binding protein. The spondylarthritides

Wright SD
van der Paardt M
Zeidler HK
Schlosstein L
Brewerton DA

Lipopolysaccharide signaling.


 Toll/interleukin-1 receptor signaling pathway in innate immune cells.


NOD2 susceptibility alleles to Crohn’s disease.


Crohn disease.


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