CONCISE REPORT

TLR4 mutations (Asp299Gly and Thr399Ile) are not associated with ankylosing spondylitis

R Adam, R D Sturrock, J A Gracie

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Background: Immunoregulatory genes and Gram negative gut bacteria are thought to be important in disease expression in ankylosing spondylitis (AS).

Objective: To compare the frequency of two common and functional TLR4 mutations (Asp299Gly, and Thr399Ile) between patients with AS and HLA-B27 healthy controls.

Methods: The TLR4 genotypes of patients and healthy HLA-B27 controls were determined using allele-specific PCR and restriction fragment length polymorphism analysis. Asp299Gly genotype was determined in 193 patients and 125 HLA-B27 positive controls and Thr399Ile genotype in 184 patients and 113 HLA-B27 controls. Allele frequencies were compared using a χ^2 test of association.

Results: 29/193 (15%) patients with AS had a polymorphism in the Asp299 site compared with 18/125 (14.4%) healthy HLA-B27 controls. Of the patients genotyped for the Thr399Ile allele, 29/184 (15.8%) carried the polymorphism compared with 19/113 (16.8%) HLA-B27 controls. No significant difference between the frequencies of the Asp299Gly genotype or the Thr399Ile genotype between patients with AS and healthy HLA-B27 controls was found. No significant difference in allele frequency was found at either site.

Conclusion: Two common TLR4 polymorphisms, which cause a functional deficiency in host immune response to Gram negative bacteria, are not overrepresented in patients with AS.

Ankylosing spondylitis (AS) is a chronic systemic rheumatic disorder that is characterised by sacroiliitis and a variety of extra-articular manifestations. Despite having the strongest association ever described with a tissue antigen, HLA-B27, the pathogenesis of AS remains poorly understood. One hypothesis is that AS occurs when Gram negative gut bacteria interact with HLA-B27 and other immunoregulatory genes. Over recent years, the importance of the innate immune system in initiating and mediating host immune response to pathogens has been recognised.

Cells of the innate immune system contain germline encoded receptors, which are genetically predetermined to focus on highly conserved microbial structures, referred to as pathogen associated molecular patterns. These receptors are known as Toll-like receptors (TLRs). Toll-like receptor 4 (TLR4) is the receptor for lipopolysaccharide (LPS), which mediates many of the inflammatory effects of Gram negative bacteria. LPS binds to TLR4 in association with three other molecules: LPS binding protein, MD-2, and CD14, and activates the nuclear factor- κ B (NF- κ B) signalling pathway.

Signalling through NF- κ B results in the production of cytokines, including interleukin 12 and interleukin 18, which can drive naïve T cells to differentiate into Th₁ cells, resulting

in the release of more proinflammatory cytokines. Another effect is the up regulated expression of CD80 and CD86 molecules on the surface of antigen presenting cells. These molecules provide costimulation for T cells, without which the T cell cannot be activated. Thus TLRs provide a link between innate and adaptive immunity.

A number of polymorphisms have been described in the genes encoding TLR4. Two cosegregating missense mutations, Asp299Gly and Thr399Ile, can affect the composition and structure of the extracellular domain of TLR4.¹ These polymorphisms cause a blunted immune response to Gram negative bacteria, with diminished levels of proinflammatory cytokines.²

It is now appreciated that TLRs, including TLR4, have a crucial role in gut immunity.³ There has been a longstanding association between macroscopic and histological gut inflammation and AS.⁴ Crohn's disease (CD) and ulcerative colitis (UC) can both cause a peripheral arthritis, and some patients go on to develop AS.

Genes encoding for receptors for the innate immune system are important in inflammatory bowel disease. It is known that mutations in the cytosolic receptor NOD2/CARD15 are associated with CD.⁵ More recently, Franchimont *et al* reported a significant association between the Asp299Gly TLR4 polymorphism and CD and UC.⁶ Torok *et al* showed that the Thr399Ile TLR4 mutation was significantly increased in patients with UC, and that the Asp299Gly and Thr399Ile mutations were increased in CD but without reaching statistical significance.⁷

Interestingly, the significance of mutations in innate immune receptors depends upon ethnic background.^{8–10}

Asp299Gly and CD14-C260T (a co-receptor) polymorphisms have been investigated in a Dutch population of patients with AS and compared with ethnically matched but not B27 matched unrelated controls. van der Paardt *et al* found no evidence that these polymorphisms were involved in AS.¹¹ This study aimed at expanding current knowledge by comparing the frequency of the two functional TLR4 polymorphisms Asp299Gly and Thr399Ile in patients with AS and HLA-B27 positive healthy controls.

PATIENTS AND METHODS

Subjects

The patients were all attending a dedicated AS clinic at the Centre for Rheumatic Diseases. A database of patients with AS exists from whom blood has been taken and DNA stored for research. For this study, DNA was used from both the existing bank, and from blood taken from new patients attending the clinic. Ethics committee approval was secured for this work.

Abbreviations: AS, ankylosing spondylitis; CD, Crohn's disease; LPS, lipopolysaccharide; PCR, polymerase chain reaction; TLRs, Toll-like receptors; UC, ulcerative colitis

Figure 1 Distribution of the TLR4 genotype at both polymorphic sites is almost identical in patients with AS and in HLA-B27 controls.

Patients were examined by a clinician and fulfilled the modified New York criteria¹² for the diagnosis of AS. At the time of enrolment, a detailed clinical history was recorded by the physician, including details of the age of disease onset, family history of AS, and extraspinal manifestations. Every patient in this study had previously been tissue typed by the Department of Tissue Typing, Glasgow Royal Infirmary, using a sequence specific primer polymerase chain reaction (PCR) method and were known to be HLA-B27 positive.

The control group consisted of blood donors who had previously been tissue typed as HLA-B27 positive by Blood Transfusion Services, Scotland. At the time of sample collection, a general health questionnaire was completed and used to determine if the person was suitable to be taken as a healthy control.

Laboratory investigation

The Asp299Gly and Thr399Ile mutations consist of single nucleotide polymorphisms in the coding region of the gene. PCR primers (Invitrogen Life Technologies) are designed with a single nucleotide alteration in the forward primer sequence that generates a restriction enzyme site in the mutant allele. Thus, distinction of wild-type and mutant alleles was possible, based on the presence of a restriction enzyme site in the mutant allele.

In the Asp299Gly, an NcoI site was created in the mutant allele, and a HinfI restriction site was created in the Thr399Ile mutant allele. The cut alleles are shorter than uncut (wild-type) alleles, and thus the alleles were separated by gel electrophoresis, and compared with a 100 base pair ladder (Bioline Ltd).

DNA from 193 patients was examined at the Asp299Gly position and compared with DNA from 125 HLA-B27 positive healthy controls. The Thr399Ile site was examined in 184 patients and compared with 113 HLA-B27 positive healthy controls.

Statistical methods

Differences in genotype and allele frequencies were analysed using a χ^2 test, with $p < 0.05$ taken as being significant. The use of over 100 patients and HLA-B27 controls allowed for an approximate power of 80% to detect a change of 15 percentage points in allele frequency, assuming a control allele frequency of at least 80%. All statistical analyses were done using Minitab, version 10.

RESULTS

Asp299Gly TLR4 polymorphism

Of 193 patients examined at the Asp299 position, 27 were heterozygous for the Asp299Gly TLR4 allele, and two were homozygous. These were compared with 125 HLA-B27 controls, 17 of whom were heterozygous for the Asp299Gly TLR4 allele, and one who was homozygous. Figure 1 shows the percentages of Asp299Gly genotypes. No significant difference was found in the frequency of the Asp299Gly TLR4 genotype ($p = 0.99362$) or allele frequency ($p = 0.989$) between patients with AS and healthy HLA-B27 controls.

Thr399Ile TLR4 polymorphism

Of 184 patients genotyped for the Thr399Ile allele, 28 were heterozygous for the polymorphism and one patient was homozygous. Of 113 HLA-B27 controls tested at this site, 18 were heterozygous for the polymorphism and one was homozygous. Figure 1 shows the percentages of Thr399Ile genotypes. No significant difference was found in the frequency of the Thr399Ile TLR4 genotype ($p = 0.9752$) or allele frequency ($p = 0.9801$) between patients with AS and healthy HLA-B27 controls.

DISCUSSION

This study is the second evaluation of the potential role of the TLR4 gene in susceptibility to AS. In contrast with the study of van der Paardt *et al*,¹¹ we analysed a larger sample of patients with AS and genetically matched HLA-B27 controls. The genotype of each subject at two polymorphic sites was determined. No evidence was found to support a significant association between the presence of Asp299Gly and Thr399Ile polymorphisms and a diagnosis of AS.

Given that TLRs have such a central role in immune system function, it is clear that they could be important in the pathogenesis of a wide spectrum of inflammatory and autoimmune diseases. The Asp299Gly allele has been identified as a protective factor in atherosclerosis, which is consistent with the fact that atherosclerosis is an inflammatory disease.⁶ However, it is worth noting that TLR mutations have not been shown to be important in many diseases with major inflammatory and infectious components, including asthma¹³ and meningococcal disease.¹⁴ A different TLR may be important in the pathogenesis of AS. Ten human TLRs have been described so far. This study looked at the LPS

receptor, TLR4, because of the association between AS and *Klebsiella*.

In summary, the data obtained show that two common functional polymorphisms of the TLR4 gene are not over-represented in patients with AS. However, given that the nature of a causal organism in AS remains uncertain, it is feasible that other members of the TLR family might contribute to an immune response against the pathogen, such as TLR9, which recognises bacterial CpG DNA.

Although susceptibility to the development of AS is not linked to TLR4 mutations, it is possible that these mutations might influence disease progression. A comparison of the TLR4 genotype and the clinical pattern of disease expression, considering features such as age of onset and the presence of extra-articular manifestations, would be a valid area for further study.

Authors' affiliations

R Adam, Centre for Rheumatic Diseases, University Department of Medicine, Queen Elizabeth Building, Glasgow Royal Infirmary University Trust, 10 Alexandra Parade, Glasgow G31 2ER, UK

R D Sturrock, J A Gracie, Section of Rheumatology, Division of Immunology, Inflammation and Infection, Faculty of Medicine, University of Glasgow, Glasgow, UK

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Correspondence to: Dr R Adam, RosalindA@doctors.org.uk

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