

HLA class I associations of ankylosing spondylitis in the white population in the United Kingdom

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Abstract

Objective—To investigate the HLA class I associations of ankylosing spondylitis (AS) in the white population, with particular reference to HLA-B27 subtypes.

Methods—HLA-B27 and -B60 typing was performed in 284 white patients with AS. Allele frequencies of HLA-B27 and HLA-B60 from 5926 white bone marrow donors were used for comparison. HLA-B27 subtyping was performed by single strand conformation polymorphism (SSCP) in all HLA-B27 positive AS patients, and 154 HLA-B27 positive ethnically matched blood donors.

Results—The strong association of HLA-B27 and AS was confirmed (odds ratio (OR) 171, 95% confidence interval (CI) 135 to 218; $p < 10^{-99}$). The association of HLA-B60 with AS was confirmed in HLA-B27 positive cases (OR 3.6, 95% CI 2.1 to 6.3; $p < 5 \times 10^{-5}$), and a similar association was demonstrated in HLA-B27 negative AS (OR 3.5, 95% CI 1.1 to 11.4; $p < 0.05$). No significant difference was observed in the frequencies of HLA-B27 allelic subtypes in patients and controls (HLA-B*2702, three of 172 patients *v* five of 154 controls; HLA-B*2705, 169 of 172 patients *v* 147 of 154 controls; HLA-B*2708, none of 172 patients *v* two of 154 controls), and no novel HLA-B27 alleles were detected.

Conclusion—HLA-B27 and -B60 are associated with susceptibility to AS, but differences in HLA-B27 subtype do not affect susceptibility to AS in this white population.

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The strong association between HLA-B27 and ankylosing spondylitis (AS) has been known since 1973.^{1,2} However, despite many advances in our understanding of the molecular biology of HLA class I molecules in general, and of HLA-B27 in particular, a comprehensive explanation for this association remains elusive. Molecular mimicry between bacterial antigens and HLA-B27 has been invoked by some as the means by which pathological autoreactive immune responses might be initiated, while others favour the presentation of 'arthritogenic peptides' specifically bound by HLA-B27. A series of amino acid substitutions clustered around the antigen binding site generate at least nine HLA-B27 allelic variants. These could influence susceptibility

to AS either by altering antigenic epitopes on the HLA-B27 molecule itself, or by affecting the range of peptides that these HLA-B27 variants bind. It is therefore of particular interest that two alleles, HLA-B*2706 in Thailand³ and HLA-B*2703 in the Gambia,⁴ appear not to be associated with AS. In contrast, the commonest alleles in white populations (HLA-B*2705 and -B*2702) and the oriental variant (HLA-B*2704) are all associated with AS,³ though it has not been formally established whether the attributable risk is identical for each of these alleles. In recent years, it has been suggested that HLA-B60 also is associated with AS, increasing the risk of disease threefold, but only in individuals who are also HLA-B27 positive;^{5,6} however, this has not been a universal finding.^{7,8}

We have investigated a large population of white patients with AS from the United Kingdom to confirm the role of HLA-B60. In addition, we have used the technique of single strand conformation polymorphism (SSCP) to define HLA-B27 alleles in the population, looking for evidence of differential association of AS with the various HLA-B27 alleles. This technique has the added potential for defining novel alleles, and has not previously been applied to typing large populations of HLA-B27 positive individuals.

Subjects and methods

PATIENTS AND CONTROLS

We recruited 284 white patients fulfilling the New York criteria for the diagnosis of AS.⁹ The HLA-B60 and -B27 phenotype and inferred genotype frequencies among 5926 white bone marrow donors (typed by the National Institutes of Health microlymphocytotoxicity assay) were used in the comparisons. In addition, 154 HLA-B27 positive, ethnically matched blood donors were identified as controls for the HLA-B27 subtype analysis. They were not known to have AS, but did not undergo specific radiological screening.

HLA TYPING FOR HLA-B27 AND -B60

DNA was extracted from frozen whole blood by the standard phenol-chloroform-proteinase K method. The presence of HLA-B27 was detected by polymerase chain reaction sequence specific primers (PCR/SSP)¹⁰ using the primers HLA-B27L (5'-CTCCATGAGGT-ATTTCCAC-3') and HLA-B27R (5'-CTG-TGCCTTGGCCTTGCA-3') (fig 1). HLA-B60 typing was also performed using

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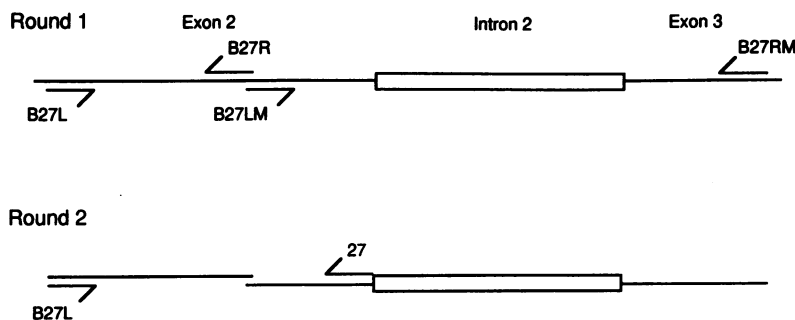


Figure 1 Seminested HLA-B27 specific polymerase chain reaction amplification. Second round product is HLA-B27 specific and encompasses all of exon 2, except the initial 5' seven base pairs.

PCR/SSP, using the primers HLA-B60L (5'-AGATCTCCCAGCGCAAGTT-3') and HLA-B60R (5'-TCAGCGCGCTCCAGC-TTG-3'). These amplify a 127 base pair segment of exon 3 of the HLA-B60 gene. A 101 base pair fragment of the α_1 antitrypsin gene was amplified as a positive control in each sample for both HLA-B27 and HLA-B60 typing, using primers PIL (5'-GCTGTTAGATTGGGTGTC-3') and PIR (5'-TAGATCAGCAGGGCATGG-3'). The reaction mix consisted of potassium chloride 50 mmol/l, gelatin 0.01%, TRIS-hydrochloric acid (1 mol/l, pH 8.3) 0.5 μ l, magnesium chloride 1.2 mmol/l, formamide 2.5%, 200 μ mol/l each of guanine, adenine, cytosine, and thymine triphosphates, 1 μ mol/l of each primer, 200 ng of DNA template, Amplitaq 2 U (Perkin-Elmer Cetus), and distilled water to a volume of 50 μ l. The reaction mix was then overlaid with mineral oil. A hot start procedure was used, followed by 28 cycles of denaturation (94°C for one minute), annealing (52°C for one minute), and extension (72°C for 30 seconds).

ANALYSIS OF HLA-B27 ALLELES

HLA-B27 subtyping was performed on the first 172 HLA-B27 positive AS patients and all HLA-B27 positive controls, by SSCP. To obtain pure HLA-B27 specific amplification product encompassing nearly all of exon 2, a two round, three amplification, seminested method was used (fig 1). Exon 2 was chosen for study because it contains most of the known polymorphisms in the HLA-B27 gene.

In round 1, two HLA-B27 specific reactions were used to amplify a 208 base pair fragment spanning exon 2 (primers HLA-B27L and HLA-B27R), and a 370 base pair fragment spanning the tail of exon 2, intron 2, and part of exon 3 (primers HLA-B27RM (5'-GGCC-CCACGTCGACGCCATACATAT-3') and HLA-B27LM (5'-GAGACACAGATCTG-CAAGGCCAAGG-3')). The reaction mix and cycling conditions for the first reaction were as above. The reaction mix for the second reaction consisted of 5 μ l of buffer (TRIS-hydrochloric acid 0.67 mol/l (from 2 mol/l pH 8.8 stock), ammonium sulphate 0.166 mol/l, beta-mercaptoethanol 0.098 mol/l), magnesium chloride 5.6 mmol/l, 200 μ mol/l each of guanine, adenine, cytosine, and thymine triphosphates, dimethyl sulphoxide 2 μ l, 1

μ mol/l of each primer, 200 ng of DNA template, Amplitaq 2 U, and distilled water to a volume of 50 μ l. A hot start procedure was followed by 28 cycles of denaturation (94°C for one minute), annealing (68°C for one minute), and extension (72°C for 30 seconds), the first cycle differing only in that the denaturation step was of five minutes duration. The HLA-B27 specific products of these amplifications were diluted 1:100, and 2 μ l of each used as the DNA template in round 2.

In round 2 the primers B27L and 27 (5'-CGGCCTCGCTCTGGTTG-3'), which are not themselves HLA-B27 specific, amplified a 263 base pair fragment. The reaction and cycling conditions were the same as for the first reaction listed above. The second round product was diluted, heat denatured, and then run in paired 10% polyacrylamide non-denaturing gels at 4°C. Samples of HLA-B27 subtypes HLA-B*2701-B*2708 were run with every pair of gels. These sequences were confirmed by DNA sequencing. Amplification products were detected by silver staining using a commercial silver staining kit (Biorad).

STATISTICAL ANALYSIS

The significance of differences in individual genotype or phenotype frequencies between patients and controls was assessed by the uncorrected χ^2 test. The cross product ratio was used to calculate odds ratios (OR), and 95% confidence intervals (CI) were computed by standard methods.¹¹

Results

HLA-B27 was present in 268 of 284 patients (94%), compared with 528 of 5926 controls (9.5%), giving an OR of 171 (95% CI 135 to 218; $p < 10^{-99}$). The phenotype frequency of HLA-B60 in the HLA-B27 positive AS patients (32 of 268, 11.9%) was significantly different from that in HLA-B27 positive controls (19 of 528, 3.6%) (OR 3.6, 95% CI 2.1 to 6.3; $p < 5 \times 10^{-5}$). The HLA-B60 phenotype frequency was significantly increased in HLA-B27 negative AS patients (three of 16, 19%) compared with HLA-B27 negative controls (333 of 5398, 6.2%) (OR = 3.5, 95% CI 1.1 to 11; $p < 0.05$).

No significant difference was observed between the frequencies of HLA-B27 allelic subtypes in patients and controls (HLA-B*2702, three of 172 patients *v* five of 154 controls; HLA-B*2705, 169 of 172 patients *v* 147 of 154 controls; HLA-B*2708, none of 172 patients *v* two of 154 controls), and no novel HLA-B27 alleles were detected.

Discussion

This study has confirmed the strength of the association of HLA-B27 and AS, and also the association of HLA-B60 and AS, though this was relatively a much weaker effect. We have also demonstrated a significant association of HLA-B60 with AS in HLA-B27 negative AS, though the number of patients studied was

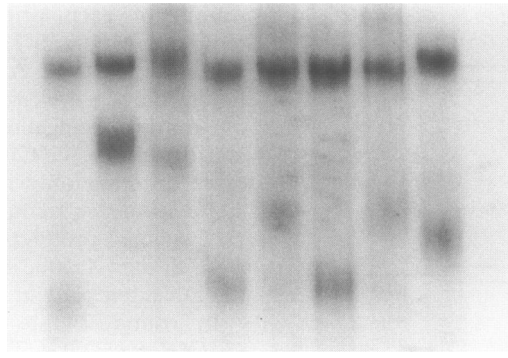


Figure 2 HLA-B*2708 subtype appearances by single strand conformation polymorphism.

small. Thus HLA-B*60 may function as a disease susceptibility gene for AS independent of HLA-B*27. The association of HLA-B*60 and AS could be attributable to a direct property of HLA-B*60, or to a further nearby linked disease susceptibility gene. HLA-B*60 might function as a restriction element for presentation of an arthritogenic peptide, or might itself provide an antigenic peptide. Similar questions remain as to how HLA-B*27 itself functions as an AS susceptibility gene.

Polymorphisms in exon 2 of HLA-B*27 are known to affect the *in vitro* function of HLA-B*27 as a restriction element.¹² Whether these *in vitro* effects have any clinical relevance is uncertain, but could account for the apparent rarity of AS in carriers of HLA-B*2703⁴ and HLA-B*2706.³ Our study had > 90% power to detect a 7% difference in the prevalence of HLA-B*2702 compared with other subtypes amongst AS patients, at a significance level of $p < 0.05$. In common with previous studies of white populations, we found no differential strength of association of the HLA-B*27 subtypes between AS patients and controls.^{8 13-16}

The study demonstrated the usefulness of single strand conformation polymorphism in analysing allelic variants in large populations. HLA-B*27 subtyping by SSCP differentiates all the known subtypes with sequence variation within exon 2 (fig 2). The technique makes it possible to divide the nine published subtypes into the groups HLA-B*2701, -B*2702, -B*2703, -B*2704/B*2706, -B*2705/B*2707/B*2709, and -B*2708. As the sequence of exon 2 is shared in HLA-B*2704 and -B*2706, and in HLA-B*2705, -B*2707, and -B*2709, this technique is unable to differentiate these subtypes. Differentiation of HLA-B*2708 from HLA-B*2705/B*2707/B*2709 is relatively difficult using this technique, and we may have underestimated the frequency of HLA-B*2708. Two of our control HLA-B*2708 subjects were initially typed as HLA-B*2705. However, previous data, based on serology, have shown that HLA-B*2708 is a comparatively rare allele, with a frequency of 0.045% in Wales,¹⁷ so any mistyping is unlikely to have affected our results significantly.

SSCP has the theoretical advantage that previously unreported subtypes that vary within the amplified region may be detected. In the PCR products of 200 or fewer base pairs, 70-95% of mutations are detected.^{18 19} Our technique is thus suitable for population screening for novel HLA-B*27 alleles, in addition to typing the major HLA-B*27 alleles. In our study, which is the largest survey of HLA-B*27 subtypes published to date, no new HLA-B*27 subtypes were detected, and no differential susceptibility to AS was evident between different HLA-B*27 subtypes.

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- Brewerton D A, Caffrey M, Hart F D, James D C O, Sturrock R D. Ankylosing spondylitis and HL-A27. *Lancet* 1973; 1: 904-6.
- Schlossstein L, Terasaki P I, Bluestone R, Pearson C. High association of an HL-A antigen W27, with ankylosing spondylitis. *N Engl J Med* 1973; 288: 704-5.
- Lopez-Larrea C, Sujirachato K, Mehra NK, et al. HLA-B*27 subtypes in Asian patients with ankylosing spondylitis. Evidence for new associations. *Tissue Antigens* 1995; 45: 169-76.
- Hill A V, Allsopp C E, Kwiatkowski D, Anstey N M, Greenwood B M, McMichael A J. HLA class I typing by PCR: HLA-B*27 and an African B*27 subtype. *Lancet* 1991; 337: 640-2.
- Robinson W, Linden S, Khan M A, et al. HLA-Bw60 increases susceptibility to ankylosing spondylitis in HLA-B*27+ patients. *Arthritis Rheum* 1989; 32: 1135-41.
- Rubin L A, Amos C I, Wade J A, et al. Investigating the genetic basis for ankylosing spondylitis. Linkage studies with the major histocompatibility complex region. *Arthritis Rheum* 1994; 37: 1212-20.
- Vargas-Alarcon G, Garcia A, Bahena S, et al. HLA-B alleles and complotypes in Mexican patients with seronegative spondyloarthropathies. *Ann Rheum Dis* 1994; 53: 755-8.
- Reveille J D, Suarez-Almazor M E, Russell A S, et al. HLA in ankylosing spondylitis: is HLA-B*27 the only MHC gene involved in disease pathogenesis? *Semin Arthritis Rheum* 1994; 23: 295-309.
- Bennett P, Wood P, eds. Ankylosing spondylitis. In: *Population studies of rheumatic diseases*, 148th edn. Amsterdam: International Congress Series; Excerpta Medica Foundation, 1966; 456.
- Cornelis F, Bardin T, Lathrop M, Bell J. Typage B27 par PCR. *Rev Rhum Mal Osteoartic* 1991; 58: 307.
- Kirkwood B. *Essentials of medical statistics*. Oxford: Blackwell, 1988; 174-6.
- Colbert R A, Rowland-Jones S L, McMichael A J, Frelinger J A. Differences in peptide presentation between B27 subtypes: the importance of the P1 side chain in maintaining high affinity peptide binding to B*2703. *Immunity* 1994; 1: 121-30.
- Aaron S, Miller M, Howard J, et al. Complementation with HLA-A and HLA-D locus alleles in ankylosing spondylitis with peripheral arthritis. *J Rheumatol* 1985; 12: 553-7.
- Appleyard J, Ahearn J, Turog J, et al. HLA-B*27 subtypes in patients with spondyloarthropathy. *Arthritis Rheum* 1992; 35: 258.
- MacLean I L, Iqbal S, Woo P, et al. HLA-B*27 subtypes in the spondyloarthropathies. *Clin Exp Immunol* 1993; 91: 214-9.
- Breur-Vriesendorp B S, Dekker-Saeyns A J, Ivanyi P. Distribution of HLA-B*27 subtypes in patients with ankylosing spondylitis: the disease is associated with a common determinant of the various B27 molecules. *Ann Rheum Dis* 1987; 46: 353-6.
- Hildebrand W, Domena J, Shen S, et al. The HLA-B*27 antigen is encoded by a new subtype of HLA-B*27 (B*2708). *Tissue Antigens* 1994; 44: 47-51.
- Michaud J. Strand-separating conformational polymorphism analysis: efficacy of detection of point mutations in the human ornithine delta-aminotransferase gene. *Genomics* 1992; 13: 389-94.
- Sheffield V, Beck J, Kwitek A, Sandstrom D, Stone E. The sensitivity of single-strand conformation polymorphism analysis for the detection of single base substitutions. *Genomics* 1993; 16: 325-32.