Ankylosing spondylitis in monozygotic twins: studies on immunological parameters

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Abstract

Objective—To examine immunological parameters that might explain disease discordance in monozygotic twin pairs with ankylosing spondylitis (AS).

Methods—11 monozygotic twin pairs (nine with AS, two with undifferentiated spondyloarthropathy) were investigated. The peripheral T cell receptor Vβ repertoire was investigated using FACS analysis and 14 different Vβ antibodies. In addition serum samples were tested for antibodies to Klebsiella pneumoniae, Streptococcus pyogenes, Candida albicans, Proteus mirabilis, and Escherichia coli. Peripheral blood lymphocyte reactivity against a number of bacteria was investigated by interferon γ ELISPOT assays.

Results—Twins suffering from AS showed cellular hyporeactivity against K pneumoniae, S pyogenes, C albicans in the ELISPOT assays compared with healthy twins. In contrast with the antibody data, where no significant differences were observed between the two groups, AS concordant twins showed the most pronounced differences in their Vβ repertoire on CD4+ and CD8+ lymphocytes.

Conclusions—Cellular hyporeactivity of peripheral blood cells to bacterial antigens might reflect defective T cell responses allowing bacterial antigens to persist in diseased patients. There are probably other environmental factors that influence disease concordance.


Ankylosing spondylitis (AS) and the other seronegative spondyloarthropathies are thought to be caused by inherited and environmental factors. Monozygotic (MZ) twins provide the unique opportunity to dissect this complex interaction. Data from a recent twin study have suggested that the development of AS is largely determined by genetic factors. Although more than 90% of AS patients carry the HLA-B27 gene, the contribution of this class I antigen to the overall disease risk is estimated to be only about 20–50%. Concordance rates among MZ twins with AS range between 50%–75% compared with 10%–15% among dizygotic (DZ) twins. A ratio of MZ twin concordance: DZ twin concordance greater than 4:1 is typical for multigenic traits. Thus it is believed that other genetic factors than HLA-B27 are involved in the pathogenesis of the disease.

However, despite the strong genetic predisposition 30–50% of monozygotic twin pairs remain discordant for AS. A number of somatic developmental events are known to modify the identical genetic background and to cause disease discordance. Rearrangements in the genes for immunoglobulin and T cell receptor loci during differentiation of the immune system constitute an important source of such differences. Other somatic factors that influence disease expression are the random inactivation of the X-chromosome and DNA methylation, which is very important in silencing certain genes.

Several lines of evidence emphasise the importance of environmental factors and especially bacterial infections in the initiation of AS. Data from patients with reactive arthritis and observations from AS patients suggest that infections with enterobacteria can trigger the onset of the disease. In patients with reactive arthritis Klebsiella pneumoniae could be isolated from the stool and increased titres of anti-K pneumoniae IgA antibodies were detected in the serum samples of patients. In addition, subclinical inflammation of the terminal ileum was observed in a large proportion of AS patients.

In this study we have investigated concordant and discordant MZ twins for differences in their peripheral T cell Vβ repertoire. Furthermore, we have tested the serum for a set of antibodies against K pneumoniae, Proteus mirabilis, and Escherichia coli and investigated whether in peripheral blood lymphocytes (PBL) there were differences in cellular reactivity against a panel of bacterial antigens.

Methods

Patients

MZ twins were sought by advertisement in the newsletter of the German Ankylosing Spondylitis Society (“Bechterew Brief”). A total of 11 MZ twin pairs responded and were invited for examination. All twins were seen and examined by the authors (TH and E M-H). Diagnosis was established according to the modified New York criteria by interview, physical examination, review of hospital records, and radiographs of the spine and sacroiliac joints. Four twins were concordant and five discordant for AS whereas in the other two twins one co-twin was affected by undifferentiated spondyloarthropathy (uSpA, patients 9.1 and 10.1 had both bilateral grade II sacroiliitis with clinical and radiological signs of spine involvement). Two healthy twin pairs unaffected by AS were used as controls (28 and 32 years old, respectively) in the investigations of the periph-
eral Vβ T cell repertoire. C reactive protein (CRP) and erythrocyte sedimentation rate (ESR) were determined in each twin when blood samples were taken. None of the twins had an increased ESR or CRP, nor did any of the patients take immunosuppressive drugs or sulphasalazine. Table 1 gives details of patient characteristics. All patients gave informed consent for the subsequent investigations. Blood samples were obtained from all twins. DNA was isolated by standard procedures. Monophygosity was determined by DNA fingerprinting analysis using the MZ1.3 multilocus probe as described in. In addition, analysis of HLA-A, -B and -C antigens was performed in every patient on PBL by the standard microlymphocytotoxicity method.\(^1\) DRB1 DNA typing was performed by nested PCR amplification using sequence specific primers.\(^12\)

**ANALYSIS OF TCR REPERTOIRE BY Vβ SEGMENT SPECIFIC MONOCLONAL ANTIBODIES**

PBL were isolated from 40 ml heparinised whole blood by standard Ficoll gradient centrifugation. Lymphocytes were washed twice with washing medium (RPMI 1640 supplemented with 5% fetal calf serum (FCS), 100 IU/ml penicillin, 100 µg/ml streptomycin) and cryopreserved in FCS (Biochrom, Berlin, Germany) with 10% dimethylsulphoxide in liquid nitrogen until analysis.

TCR-Vβ chain phenotype was ascertained by specific labelling of CD3+ T lymphocytes with monoclonal antibodies (Coulter-Immunotech, Hamburg, Germany) specific for 14 different TCR-Vβ chains, Vβ2, Vβ3, Vβ5.2, Vβ6.1, Vβ8, Vβ11, Vβ12, Vβ13.6, Vβ14, Vβ16, Vβ17, Vβ20, Vβ21.3 or Vβ22. Percentages of respective TCR-Vβ specificities of CD3+ lymphocytes were determined with a flow cytometer (FACSscan, Becton Dickinson, Heidelberg, Germany) by evaluating 10,000 CD3+ cells for each Vβ specificity. CD3+ T cells were analysed for the simultaneous expression of a specific Vβ chain and CD4 or CD8.

**ELISPOT ANALYSIS TO ENUMERATE THE PRECURSOR FREQUENCIES OF BACTERIA RESPONSIVE PBL**

To analyse the frequency of lymphocytes specific for bacterial antigens in the peripheral blood the ELISPOT technique was used as described with some modifications.\(^13\) Ninety six well microtitre plates with hydrophobic PVDF membrane bottoms (Millipore Multiscreen IP, Eschborn, Germany) were coated overnight with 100 µl/well of a solution with 10 µg/ml of a monoclonal antibody to human interferon γ (IFNγ) (Höflzel Diagnostik, Cologne, Germany) in PBS. The plates were washed four times with PBS. Triplicates of PBL (2×10⁵ cells) in RPMI 1640 medium supplemented with 10% heat inactivated human serum were added to the wells. Bacterial antigens were prepared and tested for optimal final concentrations in assay medium (RPMI-1640/10% HUS) to induce proliferative responses in PBMC bulk proliferation assays as described earlier.\(^13\) \(^14\) K pneumiae was chosen, as this pathogen has been discussed to be causally involved in the triggering of AS manifestation or AS flares.\(^9\) As control antigens, E coli was used as an enterobacterial control and S pyogenes and C albicans as other microbial recall antigens.

Positive controls were supplemented with phytohaemagglutinin (PHA, 0.5 µg/ml), negative controls with medium. After incubation for 48 hours at 37°C and 5% carbon dioxide the plates were washed four times with PBS containing 0.5% Tween. Subsequently the plates were incubated with a biotin labelled anti-IFNγ monoclonal antibody at a concentration of 6 µg/ml in PBS supplemented with 0.5% Tween. Detection
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was carried out using the Vectastain Elite ABC Kit (Vector Laboratories, Burlingame, California, USA). Spots were automatically enumerated using an electronic computer assisted imaging system (Leitz, Wetzlar, Germany). In addition, the results were checked by eye using a dissection stereomicroscope (Zeiss SV-6, Oberkochen, Germany). All results were expressed as means of triplicates from numbers of spot forming cells (SFC) per 2×10⁶ PBL after deduction of medium results.

STATISTICAL ANALYSIS

To obtain quantitative information on differences between discordant and concordant sibling pairs, we performed exploratory comparisons both within sibling pairs and among sibling pairs for each of the Vβ expressions, ELISPOT, and antibody ELISA data by one sample Wilcoxon tests.

For each of the Vβ antigens these tests were based on the relative difference in Vβ measurements, where the difference was computed for each of the sibling pairs, respectively.

To account for possibly extreme discordance the corresponding one sample Wilcoxon tests were additionally based on the absolute difference of measurements within each sibling pair.

For each of the Vβ measurements the difference in one sibling pair was compared with the corresponding differences among the subgroup of the remaining sibling pairs via one sample Wilcoxon tests (subgroups were the two healthy twin pairs, seven discordant, and the four AS concordant twin pairs). For each Vβ measurement a sibling pair was regarded significant from the others as soon as its corresponding “single Wilcoxon” p value turned out less than 0.05; the number of significant tests was then related to the overall number of tests performed in this setting. The resulting rates were then compared by χ² tests.

Results

Table 1 shows patient characteristics. Our study group comprised of six female and five male twin pairs. Nine twin pairs suffered from AS whereas in two female pairs disease had to be classified as uSpA. The mean age of the 11 twin pairs was 49.1 years. All but one discordant twin pair were well beyond the typical age of onset between 20 and 30 years. The overall concordance rate for AS was 44.4%. Interestingly concordance rates varied according to sex. Among the four female twin pairs with AS it was only 25% whereas in the five male twin pairs 60% were concordant for the disease. However the number of investigated twin pairs was too small to reach statistical significance. A previous study had suggested that B60 or DR1 influence the concordance rate among dizygotic twins. Interestingly B60 was not observed at all in our study. DR1 was present in two of the 11 twin pairs.

Vβ repertoire of peripheral T cells

None of the investigated Vβ families showed a significant variation in association with the disease status of the twins. The four discordant twin pairs showed the strongest discordance in the Vβ repertoire in relation to the number of comparisons made. Significant differences were observed in 28 of 56 comparisons (50%) for CD4+ T cells in concordant twin (4 concordant twin pairs, 14 Vβ families tested) compared with 27 of 98 comparisons (27.5%) between discordant twins (7 discordant twin pairs, 14 Vβ families tested; p<0.005).

Figure 1 Differences in Vβ repertoire in CD4+ and CD8+ T lymphocytes in the different study groups. Concordant twin pairs are shown in black, discordant twins in white and the healthy twins in grey. The y axis gives the percentage of significant differences between discordant and concordant sibling pairs. Concordant twin pairs were also concordant for AS. Fifty per cent of the remaining sib pairs were concordant for AS and 49.1 years. All but one discordant twin pair were well beyond the typical age of onset between 20 and 30 years. The overall concordance rate for AS was 44.4%. Interestingly concordance rates varied according to sex. Among the four female twin pairs with AS it was only 25% whereas in the five male twin pairs 60% were concordant for the disease. However the number of investigated twin pairs was too small to reach statistical significance.

Antibody concentrations are expressed as enzyme immunoassays units (EU): 1 EU is 1/100 of the corresponding antibody concentration in the positive reference serum.

ELISAS FOR BACTERIA SPECIFIC ANTIBODIES IN THE SERUM

Sodium dodecyl sulphate (SDS) extracts of K pneumoniae strains 21, 43, and ATCC 27736, E coli, and P mirabilis were prepared as previously described. IgM, IgG, and IgA class antibodies were measured as described earlier. The polystyrene microtitre plate (Nunc, Roskilde, Denmark) were coated with SDS extracts of K pneumoniae, E coli or P mirabilis (5 µg/ml) in PBS (0.1 mol/l, pH 7.5;100 µl/well) overnight at 37°C. The plates were saturated with 1% BSA in PBS (100 µl/well). Patients serum samples at 1:250 (IgM, IgA) or 1:300 (IgG) dilution (75 µl/well) were incubated on the plates for two hours at 37°C. Thereafter, 75 µl/well of alkaline phosphatase conjugated swine anti-human IgM, IgA or IgG (Orion Diagnostica, Espoo, Finland) diluted 1:250, 1:250, 1:500, respectively were incubated on the plates overnight at room temperature. Fresh p-nitrophenyl phosphate in diethanolamine-MgCl₂-buffer solution (1 mg/ml; Orion Diagnostica) was added, incubated for 30 minutes at 37°C and the reaction stopped with 1 M sodium hydroxide. The optical density was measured with Titertek Multiscan Photometer (Labsystems, Helsinki, Finland) at wavelength of 405 nm. Antibody concentrations were measured as described. All computations were performed using standard procedures in SAS (Release 6.10).
CD4+ T cells (p<0.005 compared with discordant twins, p<0.009 compared with healthy twins). In the discordant twins significant differences were observed in 27.5% (27 of 98) of the comparisons for CD4+ and in 34.7% (34 of 98) of the comparisons for CD8+ T cells compared with 17.9% (5 of 28) and 21.4% (6 of 28) in the two healthy twin pairs (fig 1).

**Discussion**

We have studied genetic and environmental factors that have previously been shown to be related to the development of AS in a group of MZ twins with AS and uSpA. In contrast with recent studies1 2 the overall concordance rate in our study for AS was only 44%. This number differed between male and female twins. In 60% of the male twins both were affected by AS compared with only 25% of the female twins. Although AS is a disease that predominantly affects men, women constitute 20%–40% of AS patients. If these findings could be confirmed in a larger series of twins they would suggest that female sex could have a protective effect against the development of AS.

T lymphocytes are thought to be the most important players in the immunopathogenesis of AS.4 Synovial fluid derived CD4+ and CD8+ clones recognising enterobacterial and self antigens in patients with AS have been identified.15 17 Analyses of the TCR Vβ use of HLA-B27 restricted bacteria specific and autoreactive CD8+ T-cell clones showed the preferential rearrangement of three closely related Vβ families (TCR Vβ 13, 14, 17).18 Gene rearrangements at the T cell receptor loci constitute an important somatic event that could modify a person’s susceptibility to autoimmune disease. The peripheral T cell repertoire is shaped by positive and negative selection directed by self major histocompatibility complex bearing cells in the thymus. Previous studies have shown that MZ twins are very similar in their peripheral TCRVβ repertoire, even if analyses were performed at different time points.19 20 Similar results were obtained for the two healthy concordant twin pairs in our study (fig 1). The most pronounced differences in the TCRVβ repertoire were present among the AS concordant twins in CD4+ cells and to a smaller degree for CD8+ T cells resulting in significant differences for the comparison of discordant with healthy twin pairs. Significant differences in the TCR Vβ use were also more frequent in AS discordant twin pairs compared with the healthy twins but did not reach statistical significance. These results suggest that the observed differences are most probably caused by the ongoing inflammatory process by continuous stimulation of T cells bearing different V-β families. This is supported by our own observations. Molecular analysis of the T cell receptor variability of peripheral T cells by complementarity determining region 3 assays in the investigated twin pairs has shown that clonal expansions of CD4+ and CD8+ T cells
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accounted for most of the differences observed and were much more common when both twins where suffering from AS.\(^2\) It is believed that enterobacteria, and namely \(K\) pneumoniae can trigger AS. In patients developing AS, both T cell and humoral immune responses to \(K\) pneumoniae differ from those seen in healthy people. It has been shown that anti-\(K\) pneumoniae IgA1 and IgA2 antibodies are found with increased titres in patients with AS both with and without peripheral joint disease.\(^6\) Although twins suffering from AS in general showed higher anti-\(K\) pneumoniae IgA titres differences did not reach statistical significance possibly because of the small number of people tested. The only significant difference was observed for \(K\) pneumoniae KL143 IgG antibodies, which were increased in the healthy co-twins. However, it has to be kept in mind that 18 different antibodies were tested and that borderline significant results can arise by chance.

A quantitative reduction of \(K\) pneumoniae responsive T cells in the peripheral blood of AS patients compared with healthy controls has been reported earlier by our group.\(^1\) However, it is unclear whether this reflects the ongoing disease process or a defective cellular defence against \(K\) pneumoniae secondary to HLA-B27 or other genes as suggested in several studies.\(^3,\)\(^4\) Our results from ELISPOT assays, which have been shown to be a reliable technique to enumerate the number of antigen specific T cells in the peripheral blood\(^4\) show that there are significantly less IFN\(\gamma\) secreting cells in response not only to \(K\) pneumoniae antigens but also to \(S\) pyogenes and \(C\) albicans antigens in twins with AS and uSpA compared with their unaffected twin partners. IFN\(\gamma\) is mainly secreted by T lymphocytes and to a lesser extent by natural killer cells upon stimulation. Interestingly infections with streptococci are known to trigger guttae psoriasis. The lowest reactivity towards streptococci was observed in patient 7.1, who was also suffering from psoriasis. No differences were found for \(E\) coli extracts.

In all twins antibody and ELISPOT tests were positive but there were notable differences in their individual responses. PBL of diseased twins did not not only contain less klebsiella specific IFN\(\gamma\) secreting T cells than those of their healthy twin partner, a comparable reduction was also observed for candida and \(S\) pyogenes specific responses. These findings do not strengthen the hypothesis of an involvement of a triggering klebsiella infection followed by sequestration of klebsiella specific T cells at the site of inflammation. Instead they point to a more generalised immunological hyporesponsiveness in SpA patients including other common bacterial and fungal antigens. A primarily defective first line of defence against these bacteria seems to be an unlikely explanation as the genetically identical co-twins showed normal reactivity. Another possibility is that specific cells could have been rendered anergic because of incomplete stimulation by antigen presenting cells. Defective presentation of bacterial antigens could be another reason for hyporeactivity. It has recently been shown that phagocytosis of \(Y\) enterocolitica by HLA-B27+ monocytes reduces the expression of HLA-B27 epitopes that are important for T cell recognition.\(^25\) Crossover experiments by stimulating the T lymphocytes of the affected twin with antigen presenting cells from the healthy co-twin could help to understand this phenomenon.

The results of our twin study suggest that there have to be a number of factors protecting discordant MZ twins from the development of AS. In our investigation female sex seemed to have a strong protective effect. If we accept that enterobacteria like \(K\) pneumoniae are involved in the pathogenesis of AS, we are faced with the phenomenon that all twins in our study were positive for klebsiella antibodies or in the ELISPOTS but responded differently by means of antibody and T cell reactivity. Elucidating the mechanisms that cause the differences in reactivity to these bacteria that have to be other than genetic, might help us to understand the environmental conditions that favour disease expression.

Funding: this project was supported by a grant from the Deutsches Forschungsgemeinschaft, SFB 311, Al2 and the European Commission Biomed 2 Programme.

We are grateful to Dr Claudius U Mayer for his excellent technical assistance performing the FACS analyses of PBL. We also would like to thank Tina Lahde, Petra Everke, and Jutta Lummer for their technical support.

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Ann Rheum Dis 1999 58: 435-440
doi: 10.1136/ard.58.7.435

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