Antibodies against recombinant heat shock proteins of 60 kDa from enterobacteria in the sera and synovial fluid of HLA-B27 positive ankylosing spondylitis patients

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Abstract

Objective

To study the association of HLA-B27 with IgG antibodies to different enterobacterial HSP60s in patients with ankylosing spondylitis (AS).

Methods

IgG antibodies to 60 kDa enterobacterial HSPs were determined by ELISA in paired samples of sera and synovial fluid from 21 HLA-B27+ ankylosing spondylitis (AS) patients; and in sera from 32 HLA-B27+ AS patients, 35 HLA-B27+ healthy relatives of AS patients, and 60 HLA-B27– healthy individuals with no family members with AS.

Results

HLA-B27+ patients and healthy individuals showed significantly higher IgG antibody levels to recombinant enterobacterial HSP60s than HLA-B27– healthy controls. The levels of anti-HSP60Sf and anti-HSP60Ec antibodies correlated with disease activity and anti-HSP60Ec antibodies with male gender. No association between enterobacterial HSP60 antibody levels and disease duration was observed. All groups had lower levels of IgG antibodies to rHSP60 from Streptococcus pyogenes *(rHSP60 Spy). In paired samples of sera and synovial fluid from B27+ patients, IgG antibodies to enterobacterial HSP60s were detected, but in significantly higher levels in sera than in synovial fluid. The anti-rHSPSpy IgG response in these samples was lower and similar in the three groups.*

Conclusions

A correlation was found between HLA-B27 and the response to recombinant enterobacterial HSP60s. This response could be associated with disease activity and gender in some proteins and the presence of IgG antibodies to these proteins in synovial fluid could be associated with the inflammatory process and initiation of AS.

Key words

Ankylosing spondylitis, heat shock proteins, enterobacteria, synovial fluid, serum, antibodies.

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Introduction

The high correlation of the MHC class I antigen HLA-B27 with two closely related rheumatic diseases, ankylosing spondylitis (AS) and reactive arthritis (ReA) – with 95% and 60-80% positivity in AS and ReA, respectively (1, 2) – represents the strongest known link of any MHC molecule with any group of human diseases. Because of their similar and overlapping clinical pictures and the HLA-B27 association, these diseases have been classified, together with other HLA-B27-associated diseases, as spondyloarthropathies (3, 4). The spondyloarthropathies are documented clinically largely on the basis of joint inflammation and have been classified as rheumatic diseases.

However, a large body of evidence suggests an intimate involvement of intestinal inflammation in the pathogenesis of these diseases, *e.g.* the triggering of B27+ associated reactive arthritis by certain enteritis or urethritis pathogens such as *Salmonella, Shigella, Yersinia* and *Chlamydia trachomatis*. Moreover, an increased prevalence of ankylosing spondylitis and peripheral arthritis in patients with inflammatory bowel disease has been reported (5-7). A relationship between AS and enterobacteria, especially *Klebsiella pneumoniae*, has been reported by various groups in several countries (8). Clinical and experimental findings in patients with enteropathic spondylarthropathies are consistent with the involvement of gastrointestinal gram-negative bacterial infection in the development of the arthritic lesions. This has been demonstrated, however, only in post-enteric ReA, in which enterobacteria antigens have been found in the joint (9). More recently, bacterial DNA has been identified in synovial fluid cells of patients with juvenile or adult-onset spondyloarthropathies (10).

Ankylosing spondylitis can be considered a reactive arthritis following *K. pneumoniae* infection in HLA-B27 positive patients, based on consistent findings of raised anti-*Klebsiella* antibodies (11-13). In addition IgM, IgG and IgA antibodies to *K. pneumoniae, Escherichia coli* and *Proteus mirabilis* have been reported in sera, synovial fluid and jejunal perfusion fluid from AS patients (14, 15). IgA specific to *Klebsiella, Shigella* and *Yersinia* have been found in the serum of AS patients (16). Heat shock proteins (HSP) are a family of proteins whose expression is increased by several stimuli, of which heat is one. HSPs show a high degree of sequence homology throughout phylogenesis (17, 18) and are immunodominant in the humoral response to many infectious organisms (19). Laboratory and clinical observations in patients with autoimmune diseases indicate that immune responses to HSP arise spontaneously during the disease process (20). They may be directly immunogenic, share immunogenic epitopes with various pathogens implicated in autoimmunity, and may be structurally linked with MHC genes (21, 22). Homology between different bacterial HSP60s is very high. Moreover, various findings with regard to the humoral response to 60 kDa enterobacterial HSP and the proliferative response to HSP from *K. pneumoniae* have been reported in HLA-B27 AS patients (18, 23-25).

Recently we reported increase levels of IgG antibodies against native enterobacterial HSP60s in HLA-B27 positive AS patients and healthy individuals (26). Here we have extended our earlier studies by investigating IgG antibody levels against recombinant enterobacterial HSP60s in the serum and synovial fluid of HLA-B27 positive ankylosing spondylitis patients.

Patients and methods

Patients and controls

This study included 127 Mexican Mestizo individuals who were divided into one disease group and two healthy control groups: Group I, consisting of 32 HLA-B27+ ankylosing spondylitis patients; Group II, comprising 35 HLA-B27+ healthy relatives of AS patients, and Group III, comprising 60 healthy B27– individuals with no history of AS in their families. Among the 32 AS patients, 21 had active disease and therefore a paired synovial fluid sample was included.

The diagnosis of AS was made based on current criteria (27). We estimated a sample size of 110 that included

patients and controls based on the MacNemar test (α=0.05, β=0.2) (28). The clinical characteristics of the 32 patients are shown in Table I. Written consent was obtained from all participants. All sera and synovial fluid were stored at -70ºC until tested.

Table I. Clinical characteristics of patients with AS.

Subject	Age, Sex	Disease Onset*	Disease Duration yrs	Disease activity	Treatment
1	38, F	J	21	Inactive	Sulfasalazine
2	20, M	J	1	Inactive	Paracetamol
3	51, M	J	31	Inactive	Sulfasalazine
4	28, M	J	14	Inactive	Sulfasalazine Indomethacin
5	28, F	A	7	Active	Sulfasalazine
6	31, M	A	6	Active	Sulfasalazine
7	20, M	J	3	Active	Sulfasalazine
8	23, M	A	0.1	Active	Sulfasalazine
9	31, M	A	6	Active	Paracetamol
10	17, M	J	2	Active	Sulfasalazine Indomethacin
11	22, M	J	5	Active	Sulfasalazine
12	51, M	A	28	Inactive	Sulfasalazine
13	24, F	J	15	Active	Sulfasalazine Indomethacin
14	35, F	A	2	Active	Indomethacin
15	32, F	A	3	Active	Sulfasalazine
16	18, M	J	1	Active	Sulfasalazine
17	32, M	A	9	Active	Nimesulide
18	16, M	J	6	Active	Sodium Diclofenac
19	31, M	A	5	Active	Paracetamol
20	53, F	A	5	Active	Paracetamol
21	32, M	A	13	Active	Indomethacin Paracetamol
22	34, M	A	14	Inactive	Paracetamol
23	48, M	A	25	Active	Indomethacin
24	32, M	J	21	Active	Sodium Diclofenac
25	35, M	J	23	Active	Indomethacin
26	42, M	J	30	Active	Sulfasalazine
27	52, F	J	40	Active	Sulfasalazine
28	36, F	J	20	Inactive	Indomethacin Naproxeno
29	35, M	J	25	Inactive	Indomethacin
30	21, F	J	8	Inactive	Indomethacin
31	43, M	J	29	Inactive	Indomethacin
32	51, F	A	13	Inactive	Indomethacin Paracetamol

*Disease onset: Juvenile (J): <15 years old, adult (A): >17 years old.

Antigens

Purified recombinant 60 kDa heat shock proteins from *Klebsiella pneumoniae* (Kp), *Yersinia enterocolitica* (Ye), *Escherichia coli* (Ec), *Salmonella typhi* (Sty), *Shigella flexneri* (Sf) and *Streptococcus pyogenes* (Spy) were prepared as previously described (29). Briefly, all genes encoding the 60 kDa heat shock proteins of Kp, Ye, Ec, Sty, Sf and Spy respectively were amplified by PCR using the corresponding genomic DNA of each bacteria and appropriate primers designed according to the published gene. They were cloned in the pProEXHTa plasmid. The clones of *E. coli* XL1-Blue containing recombinant plasmids were grown at 37ºC in 100 mL liquid cultures of Luria broth supplemented with 0.6 mM isopropyl-β-D-thiogalactopyranoside (Sigma Aldrich, St. Louis MO, USA). Recombinant proteins were purified by affinity chromatography on metal-chelating Ni resin (Quiagen Inc., CA, USA) and analyzed by SDS-PAGE following the discontinuous buffer system of Laemmli (30). All recombinant proteins showed a single band with a molecular weight of 60 kDa after Coomassie blue staining.

Quantification of IgG antibodies to rHSP by ELISA

Flat-bottom polystyrene plates (NUNC Maxisorb, Denmark) were coated with the 60 kDa rHSP proteins – in each case 100 µl of rHSP at 1.0 µg/mL, diluted in 15 mM sodium carbonate-35 mM sodium bicarbonate buffer, pH 9.6 – and incubated overnight at 4ºC. After incubation the plates were washed 3 times with PBS 0.5% Tween 20 (PBS-T) and blocked with 2.5% skim milk in carbonate buffer. Human sera and synovial fluid were diluted 1:300 in PBS 2.5% skim milk and tested in duplicate, in the presence and absence of the rHSP60 antigens. The wells were then filled with 100 µl serum or synovial fluid sample, incubated for 2 h at 37ºC, and washed 6 times, as described above. Peroxidase-conjugated goat IgG anti-human IgG (Copper Biomedical Inc., West Chester, PA, USA) was diluted 1:4000 in PBS-skim milk, 100 µl was added to each well, and the plates were incubated for 1 h at 37ºC. They were then washed 6 times with PBS-T, and 100 µl of substrate solution (0.4 mg/ml o-phenylendiamine and 0.01% hydrogen peroxide in citrate-phosphate buffer, pH 5.0) was added. After 30 min the incubation reaction was stopped with 8 N sulphuric acid.

Absorbance was measured at 492 nm in an ELISA reader (Tititerk Multiskan, Flow Labs, Finland), using wells without antibody as blanks (antibody control). To compensate for non-specific "background" binding of immunoglobulins to the plate, the absorbance in the uncoated wells was subtracted from the absorbance found for the antigen-coated wells. The values were plotted in corresponding graphs. In each plate, 2 positive sera or 2 synovial fluids were always included as an internal control of reproducibility.

Statistical analysis

Data were analyzed and graphed using Graph-Pad Prism. The significance of the difference (p<0.05) between medians was calculated by the Mann-Whitney U test. The confidence interval was 95%. Correlations between serum IgG antibody levels against bacterial HSPs (dependant variables) and disease activity, sex, age and disease duration (independent variables) were evaluated by one-way analysis of variance on ranks (ANOVA), followed by multiple comparison using Dunnet's method. The correlations between IgG antibody levels with two-paired independent variables were tested using a two-way

analysis of variance following by pairwise multiple comparison procedures (Tukey Test). These statistical analyses were performed using the Sigma Stat 2.03® program. For all the statistical analyses, the minimum probability criteria for significance differences was set at $p<0.05$.

Results

IgG serum antibody levels to rHSP60

Levels of IgG antibodies to each purified bacterial recombinant HSP60 were measured by ELISA, and the individual levels of IgG antibodies against the six different bacteria for the three groups studied – HLA-B27+ AS patients, HLA-B27+ healthy relatives of AS patients, and HLA-B27– healthy individuals with no family history of AS – are shown in Figure 1.

IgG antibody levels to rHSP60 from *K. pneumoniae*, *Y. enterocolitica*, *E. coli*, *S. typhi* and *S. flexneri* (Sf) were significantly higher ($p<0.05$) in groups I and II than in group III (Fig. 1, A-E). Antibody levels to recombinant HSP60 from *S. pyogenes* were also determined and found to be significantly lower than the antibodies to the other enterobacterial rHSP60 for all 3 groups ($p<0.05$), with no significant differences observed between groups (Fig. 1F). The mean values for IgG antibody levels to each enterobacterial rHSP60 were similar in each group. In all groups the levels of IgG antibodies to enterobacterial rHSP60 were significantly higher than IgG levels to rHSP60Spy ($p<0.05$).

Serum IgG antibody levels against enterobacterial HSPs were considered to be "high" if the individual value was at least two standard deviations above that of the mean values ($As_{492\ nm}$) obtained for each protein in the HLA-B27– controls. In group I, 21–53% of the subjects had high antibody levels to each enterobacterial HSP. In group II, 11.4–48.5% had high antibody levels, and in group III 2–5% (Table II). In this analysis we observed that the serum samples from 4/21 patients with active disease (19%) showed high IgG antibody levels against all five enterobacterial HSP (data not shown).

For the analysis, the patients were divided into three groups by disease duration: 25% had AS for 0.1 to 5 years, 50% for 5.1 to 21.9 years, and 25% for 22 to 40 years. They were also divided into three groups by age: 25% were 17 to 23.5 years of age, 50% were 23.6 to 39.9 years of age, and 75% were 40 to 53 years of age. Levels of antibodies against HSP60Sf and HSP60Ec correlated significantly with disease activity ($p<0.05$). Antibody levels against HSP60Ec correlated significantly with male gender ($p<0.05$). No association between antibody levels against any of the five enterobacterial HSPs and disease duration was observed.

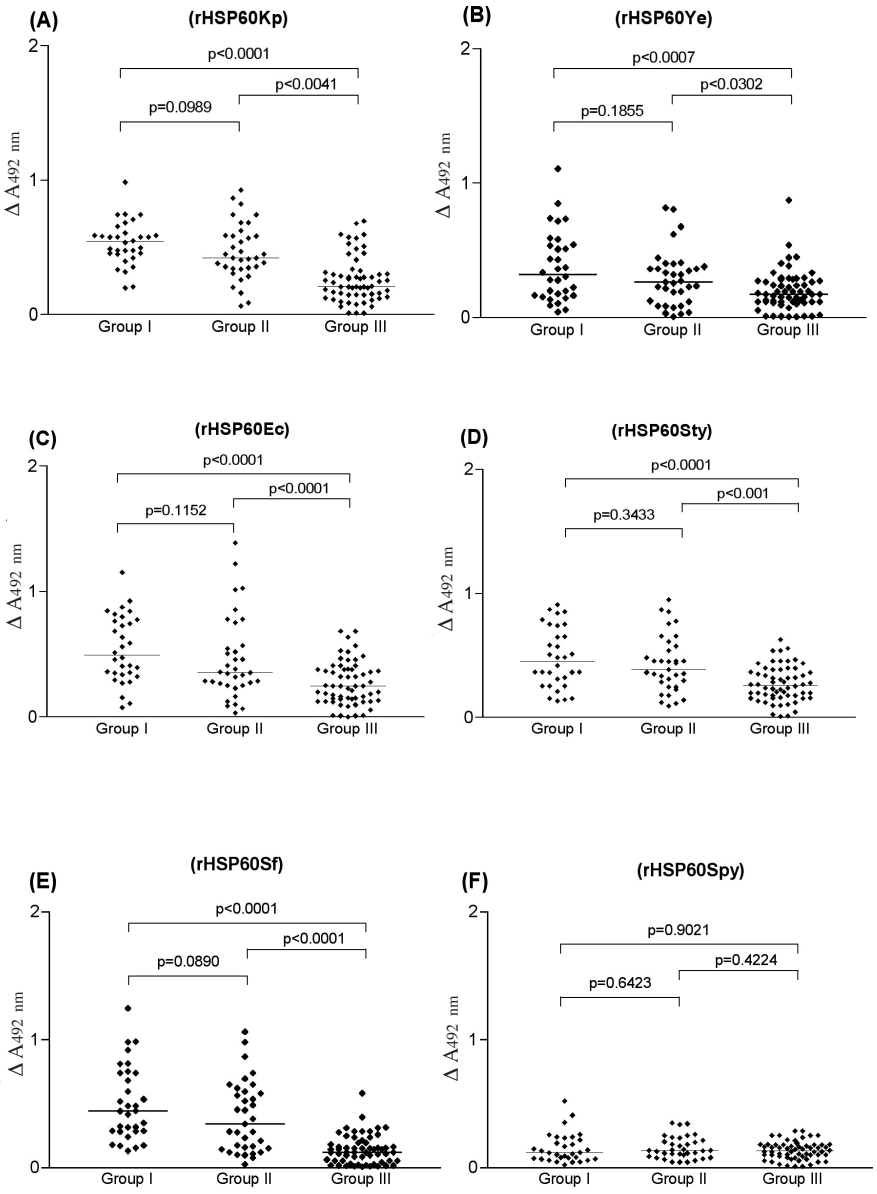

Fig. 1. Comparison of recombinant bacterial HSP60 IgG antibody levels in B27+ AS patients (Group I), B27+ healthy relatives of AS patients (Group II), and B27– healthy controls with no family history of AS (Group III). Levels of anti-rHSP60Kp (panel **A**), anti-rHSP60Ye (panel **B**), anti-rHSP60Ec (panel **C**), anti-rHSP60Sty (panel **D**), anti-rHSP60Sf (panel **E**), and anti-rHSP60Spy (panel **F**) were determined by ELISA. Sera were diluted 1:300 and each recombinant bacterial HSP60 was assayed at 0.1 μg/well. Each dot represents the mean value for 3 different determinations made in triplicate each time in one subject. The horizontal line represents the median A_{492nm} value for each group. Statistical analysis of each group compared to the others was carried out using the Mann-Whitney U test.

IgG antibodies in paired samples of sera and synovial fluid from AS patients

Levels of IgG antibodies to rHSP60 in paired samples of sera and synovial fluid from the HLA-B27+ AS patients were measured by ELISA. For five of the bacteria, the mean IgG antibody

Table II. Percentages of patients with AS B27+ (Group I); healthy subjects B27+ AS related individuals (Group II), and healthy controls HLA-B27-, non AS-related individuals (Group III) that had high serum IgG antibody levels against bacterial recombinant 60kDa heat shock proteins.

IgG antibodies against	HLA-B27negative controls		Patients with AS B27+ (n=32)	Healthy subjects B27+ AS related individuals (n=35)	Controls HLA-B27-, non AS-related individuals (n=60)
	Mean of $As_{495\ nm}$	Plus two standard deviations	(no. of patients), %		
rHSP60Kp	0.2697	0.6143	(13), 40	(9), 26	(3), 5
rHSP60Ye	0.2046	0.5032	(10), 31	(4), 11.4	(2), 3
rHSP60Ec	0.2609	0.6005	(7), 21	(8), 22.8	(3), 5
rHSP60Sty	0.2738	0.5642	(9), 28	(9), 25.7	(2), 3
rHSP60Sf	0.1452	0.3704	(17), 53	(17) 48.5	(2), 2
rHSP60Spy	0.1351	0.2752	(3), 9.3	(3), 8.5	(2), 2

The percentages represent the number of subjects that had high serum IgG antibody levels against bacterial recombinant 60 kDa heat shock proteins. We considered as a high IgG antibodies levels if the mean values $As_{495\ nm}$ was at least two standard deviation above that of mean value $As_{495\ nm}$ of HLA-B27- controls.

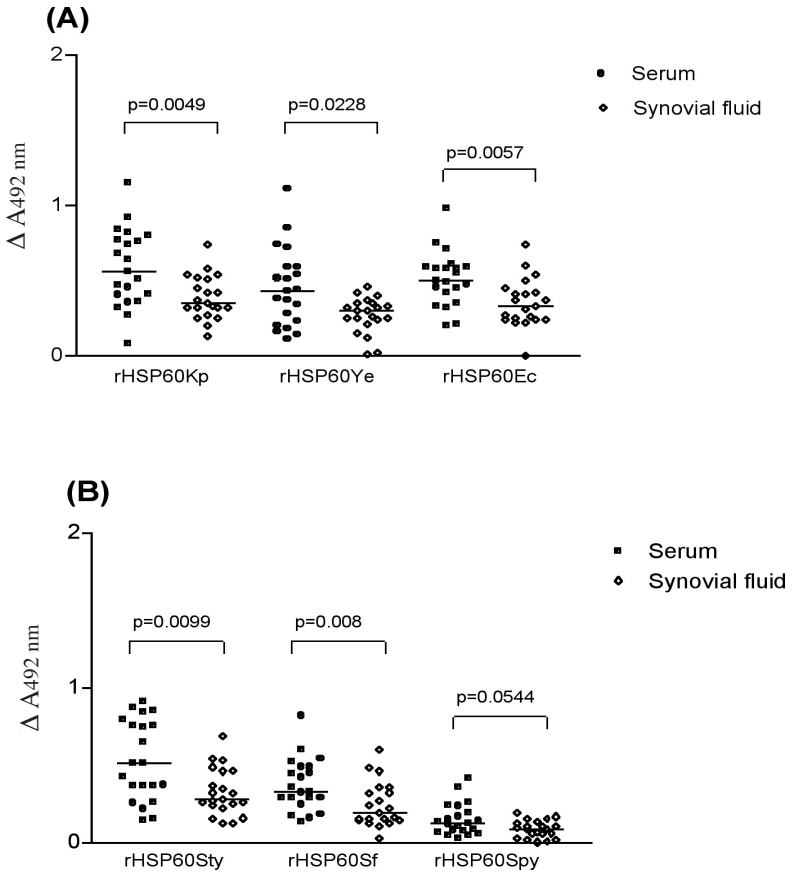

Fig. 2. Comparison of IgG antibody levels against recombinant bacterial HSP60 in 21 paired samples of serum and synovial fluid from B27+ AS patients. Levels of anti-rHSP60Kp, anti-rHSP60Ye and anti-rHSP60Ec (panel **A**), and anti-rHSP60Sty, anti-rHSP60Sf and anti-rHSP60Spy (panel **B**) were determined by ELISA. Sera and synovial fluid were diluted 1:300 and each recombinant bacterial HSP60 was assayed at 0.1 μg/well. Each dot represents the mean value for 3 separate determinations made in triplicate each time from one subject. The horizontal line represents the median A_{492nm} value for each group. Statistical analysis of each group compared to the others was carried out using the Mann-Whitney U-test.

levels to rHSP60 were significantly higher ($p<0.05$) in serum than in synovial fluid (Fig. 2, A and B); only for *S. pyogenes* were antibody levels lower in both the serum and synovial fluid, with no significant differences between them (Fig. 2B). The mean serum levels of IgG antibody to rHSP60 from *K. pneumoniae*, *Y. enterocolitica*, *E. coli*, *S. typhi* and *S. flexneri* were similar. In synovial fluid the mean values were also similar, although lower than in the sera.

Discussion

Heat shock proteins are stress-induced molecules that promote protein stability and facilitate their transport (31). Recent studies have focused on the interplay between HSP and innate and acquired immunity. In studies of the B cell response, patients with RA showed higher levels of IgG and IgA antibodies against mycobacterial HSP65 than healthy controls (32). In addition, high antibody titers against *E. coli* GroEL compared to antibodies against mycobacterial HSP65 have been reported in the sera of patients with RA and in healthy adults (33, 34). Different HSP proteins (e.g., HSP60 and HSP70) show a high degree of homology in their sequences in various pathogenic or non-pathogenic bacteria, and are known to induce very strong humoral immune responses in numerous diseases (35).

However, the titers of antibodies against HSPs can vary from patient to patient and indeed HSP-specific antibodies have occasionally been found in healthy individuals. We therefore concluded that the role of these proteins in autoimmune diseases is still not completely understood. Increased levels of HSP60 antibodies have been found in the serum of patients with atherosclerosis, systemic sclerosis (36), psoriasis (37), Kawasaki disease (38) and Behçet's disease (39), in many cases exceeding those found in healthy controls. In a rat model of adjuvant arthritis, HSP-specific antibodies with reactivity to synovial tissue similar to that seen in patients with RA has been demonstrated (40). In juvenile chronic arthritis such HSP antibodies react with synovial membranes and the expression of self-HSP60 in inflamed synovium is significantly raised

(41). It has been suggested that antibodies to mycobacterial HSP60 play a role in AS and RA, and cross reactivity to self-HSP may contribute to these disease processes (42).

In the present study, both HLA-B27+ AS patients and healthy HLA-B27+ relatives of AS patients showed significantly elevated levels of sera IgG antibodies to enterobacterial recombinant HSP60s, while HLA-B27– healthy controls exhibited lower IgG levels. It is interesting that 21–53% of B27+ patients and healthy subjects presented high serum IgG levels for each enterobacterial protein compared to 2-5% in the B27– controls. Moreover, 4 out of 21 patients with active disease (19%) showed high levels of serum antibodies against five of the enterobacterial HSPs. This could be due to the recognition of a common epitope in these HSPs, but more experiments are needed to test this hypothesis.

The homology between enterobacterial HSP60s is very high (about 60-85%) in this group, and the homology is 60% with human HSP60. These findings are in agreement with our own; in a previous study we reported a relationship between HLA-B27 and the IgG response to native HSP60s from *Klebsiella, Yersinia, Escherichia* and *Salmonella* in AS patients (26).

The serum antibody levels against enterobacterial HSPs were compared with antibody levels against the rHSP60 of *S. pyogenes*, which were significantly lower in the three groups analyzed. Although HSPs are highly conserved among bacterial species (18) and are highly antigenic, no association with streptococcal infection in AS patients has been reported; at the same time, enterobacterial infections have been associated with AS. At the time of sampling none of our patients or controls showed any symptoms of gut inflammation or Crohn-like colitis attributable to NSAID treatment. The high antibody titers detected in our B27+ patients and healthy subjects can probably be explained by a past enterobacterial infection. These findings are all the more significant because the subjects in our study could be representative of a population with a high exposure to *Salmonella, Shigella* and *E. coli* infection. Such a connection would explain the significant correlation between the high antibody levels against *Shigella* and *Escherichia* HSP60 and disease activity observed in the present study. However, the significance of this finding requires further investigation.

In this study we also analyzed IgG antibody levels to bacterial rHSP60s in paired samples of sera and synovial fluid. The results are of interest, because IgG antibodies to enterobacterial rHSP60s were found in the synovial fluid of HLA-B27+ AS patients, although at significantly lower levels than in the corresponding serum samples. This finding is consistent with the report of Pacheco-Tena *et al*., who identified *Salmonella, Shigella* and other bacterial DNA in the synovial fluid cells of patients with juvenile onset SpA (10). Other studies have demonstrated bacteria DNA in the synovial fluid or synovial membrane of patients with adult-onset reactive arthritis, Reiter's syndrome, undifferentiated oligoarthritis, and various forms of juvenile arthritis. Remarkably, bacterial DNA has also been found in patients with osteoarthritis or rheumatoid arthritis, and even in healthy controls (43, 44).

All of this data suggests that patients may be exposed to enterobacterial infections that attack the joints under stress conditions. The production of both bacterial and host HSPs is induced, initiating a specific and localized antibody response. This hypothesis would be in accord with the findings of Cunnane *et al*. (45), who showed that the synovial membrane of affected joints in ankylosing spondylitis is infiltrated by germinal center-like aggregates of B lymphocytes similar to those seen in rheumatoid arthritis. Although local antibody synthesis takes place within the joint in rheumatic diseases, the principal component of the synovial fluid immunoglobulins is plasma-derived, by increased influx through the inflamed synovial membrane (46, 47). This phenomenon could explain the higher IgG antibody levels against HSP60 detected in the sera than in the synovial fluid of AS patients.

In conclusion, high levels of IgG antibody to enterobacterial rHSP60s were found in the sera of HLA-B27+ AS patients and the healthy relatives of AS patients. It is interesting to note that a correlation was observed between serum antibody levels against HSP60Sf and HSP60Ec and disease activity, and between antibody levels against HSP60Ec and male sex. This suggests that the antibody response to enterobacterial HSP can probably be linked to the presence of the HLA-B27 molecule. The response could also be correlated with disease activity and gender for some proteins, confirming our previous results with native enterobacterial HSP60s in the sera of B27+ subjects with previous exposure to these bacteria.

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