

EXTENDED REPORT

High levels of IgG class antibodies to recombinant HSP60 kDa of *Yersinia enterocolitica* in sera of patients with uveitis

J C Cancino-Diaz, L Vargas-Rodríguez, N Grinberg-Zylberbaum, M A Reyes-López, M L Domínguez-López, A Pablo-Velazquez, M E Cancino-Diaz

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Correspondence to:
Dr M E Cancino-Diaz, San Bartolo Naucalpan, No 86 Edif N2 Depto 7 Col Argentina, Delg Miguel Hidalgo, 11270 Mexico; mcancino@encb.ipn.mx

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Aims: To determine the levels of IgG class antibodies to recombinant heat shock protein 60 kDa of *Yersinia enterocolitica* (rHSP60Ye), *Klebsiella pneumoniae* (rHSP60Kp), *Escherichia coli* (rHSP60Ec), *Shigella flexneri* (rHSP60Sf), and *Streptococcus pyogenes* (rHSP60Sp) in the serum of patients with HLA-B27 associated acute anterior uveitis (HLA-B27 associated AAU), idiopathic acute anterior uveitis (idiopathic AAU), pars planitis, Vogt-Koyanagi-Harada (VKH), and healthy subjects.**Methods:** The genes that code for HSP60Ye, HSP60Kp, HSP60Ec, HSP60Sf, and HSP60Sp were cloned by PCR from genomic DNA. The rHSPs were purified by affinity using a Ni-NTA resin. The serum levels of IgG class antibodies to rHSP60s were determined by ELISA in patients with uveitis (n=42) and in healthy subjects (n=25).**Results:** The majority of patients with uveitis had higher levels of IgG class antibodies to rHSP60Ye compared with levels of healthy subjects (p=0.01), although these differences were only observed in the HLA-B27 associated AAU (p=0.005) and in pars planitis patients (p=0.001). The levels of IgG antibodies to the rHSP60Kp, rHSP60Sf, rHSP60Ec, and rHSP60Sp were similar in patients with uveitis and in healthy subjects (p>0.05).**Conclusion:** The results suggest that HSP60Ye could be involved in the aetiology of HLA-B27 associated AAU and pars planitis.

Heat shock proteins (HSPs) are constitutive and inducible proteins that play an important role in cellular protection to stress. Recently, HSPs have been involved in the innate immune response, possibly caused by binding to the CD14-TLR4 complex of the presenting antigen cell and therefore producing an inflammatory effect.^{1–3} These proteins are highly conserved during the evolution and are expressed in all organisms. The role of HSPs in autoimmune diseases is strongly documented;^{4–5} however, their function in inflammatory eye diseases has not been extensively studied.

When uveitis is associated with systemic Behçet's disease (BD), it has been linked with microbial⁶ and human⁷ HSP60 and HSP70.⁸ Recently, it has been shown that T cells in BD can proliferate and produce inflammatory cytokines^{7–9–10} when they are stimulated with the HSP of different microorganisms such as *Mycobacterium tuberculosis*, *Streptococcus pyogenes*, and *Yersinia enterocolitica*.^{9–11–12}

In this study, we analysed the levels of IgG class antibodies of five different bacterial HSP60s in the sera of patients with idiopathic anterior acute uveitis (idiopathic AAU), HLA-B27 associated anterior acute uveitis (HLA-B27 associated AAU), Vogt-Koyanagi-Harada (VKH), and pars planitis.

PATIENTS AND METHODS

A total of 42 patients with a history of acute or recurrent uveitis were included. A visual acuity test, tonometry, slit lamp examination, and an evaluation of the fundus with indirect ophthalmoscopy and a three mirror lens when necessary were performed. Data concerning age, sex, recurrence, complications, and systemic symptoms and disorders were collected on standard forms. A rheumatological survey with clinical examination and radiological examination of lumbar spine and sacroiliac joints were conducted in patients with symptoms suggestive of ankylosing spondylitis or other spondyloarthropathies. The level of ocular inflammation was

determined by clinical examination of the increase in number of anterior chamber or vitreous cells. Uveitis was classified according to the International Uveitis Study Group.¹³ All patients were taking steroidal anti-inflammatory drugs. Controls (n=25) were healthy subjects with no previous or present ocular disease, no clinical evidence of a systemic pathology, and not positive to HLA-B27 antigen. The presence of HLA-B27 antigen was determined in patients and control subjects as described by Teresaki *et al.*¹⁴ Serum was collected from each subject and stored at –70°C until needed.

All subjects in our study were grouped as follows: group 1, healthy subjects (n=25); group 2, uveitis patients (n=42). Group 2 was subdivided according to the uveitis type as follows: group 2a, HLA-B27 associated AAU only with or without spondyloarthropathies (n=11); group 2b, pars planitis only (n=13); group 2c, idiopathic AAU only (n=8), and group 2d, VKH only (n=10).

The genes that code for heat shock protein 60 kDa of *Yersinia enterocolitica* (rHSP60Ye), *Klebsiella pneumoniae* (rHSP60Kp), *Escherichia coli* (rHSP60Ec), *Shigella flexneri* (rHSP60Sf), and *Streptococcus pyogenes* (rHSP60Sp) were cloned by PCR, using the genomic DNA of each bacterium. All genes were cloned into the pProEXHTb plasmid (Gibco, Life technology, Rockville, MD, USA) and recombinant proteins were purified by Ni-NTA resin (Qiagen Inc, Valencia, CA, USA).

Indirect ELISA was done to measure the antibody levels of the different rHSP60s studied. Wells were coated with 0.5 µg of the recombinant proteins. After overnight incubation at 4°C, the plate was washed and 100 µl of the sample dilutions (1:300) were added in each well and incubated for 1 hour.

Abbreviations: AAU, acute anterior uveitis; BD, Behçet's disease; HSP, heat shock protein

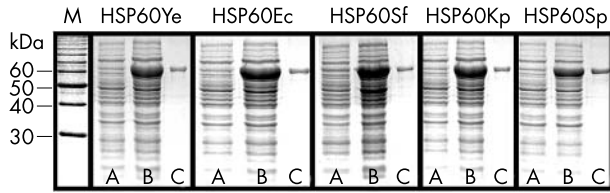


Figure 1 Purity analysis of the recombinant HSPs in PAGE. Protein extracts of *E. coli* transformed with recombinant plasmid not induced (A) and induced with 0.6 mM of IPTG (B). (C) Purified rHSP60 with Ni-NTA resin. Molecular weight marker (M).

After washing, the plates were incubated with 100 µl peroxidase conjugated goat IgG anti-human IgG (Copper Biomedical Inc, West Chester, PA, USA) for 1 hour, followed by addition of H₂O₂ and o-phenylene-diamine as substrate. Absorbance was measured at 492 nm.

Statistical analysis of the data was performed using Mann-Whitney U test to compare groups. The odds ratio was used for determine the relation between levels of antibody and the disease.

RESULTS

In order to study the humoral immune response of patients with uveitis to HSPs, genes that code for HSP60Ye, HSP60Kp, HSP60Sf, HSP60Ec, and HSP60Sp were cloned. The purity analysis of the five rHSP60s is shown in figure 1.

The levels of IgG class antibodies to HSP60 present in the sera of the patients are shown in figure 2. We found that the majority of the patients with uveitis (group 2) had higher antibody levels to rHSP60Ye than healthy subjects (group 1; p = 0.01), but these differences were only found in the group of patients with HLA-B27 associated AAU (group 2a; p = 0.005) and in the group of patients with pars planitis (group 2b; p = 0.001). The antibody levels of the groups of patients with idiopathic AAU (group 2c) and VKH (group 2d) were similar to those shown by healthy subjects (p>0.05), (fig 2A).

The antibody levels to the others enterobacterial HSPs studied (rHSP60Ec, rHSP60Sf, and rHSP60Kp) in the sera of all patients with uveitis (group 2) were not different when compared with the levels of healthy subjects (group 1; p>0.05). Similar results were obtained when the antibody levels were compared with the subgroups (p>0.05) (fig 2B, C, and D). The antibody levels to rHSP60Sp (used as a Gram positive, non-enterobacterial control) were similar in all the

groups studied (p>0.05) (fig 2E), but these were lower than the antibodies levels observed from the enterobacterial rHSP60s (p<0.05).

To determine the degree of association between the humoral immune response to rHSP60Ye and the disease, we calculated the odds ratio value. To calculate the odds ratios we arbitrarily considered A_{492nm} greater than 0.58 (A_{492nm} mean+1 SD of the control subjects) as a positive response to rHSP60Ye, and values less than 0.58 as negative. The odds ratio value for HLA-B27 associated AAU patients was 6 (95% CI 1.22 to 29.73). For pars planitis patients the odds ratio was 5.8 (95% CI 1.26 to 26.94). The odds ratios were not significant for all diseases studied with the other HSP60s assayed.

With regard to the relation between positive IgG response to rHSP60Ye and clinical characteristics of the HLA-B27 associated AAU and pars planitis patients, we found that sex, age, recurrence, level of inflammation, and spondyloarthropathy were evenly distributed between the patients with positive and negative IgG response to rHSP60Ye. However, eye complications were observed more often in the HLA-B27 associated AAU patients with positive IgG response to rHSP60Ye only (p = 0.0358, table 1). The mean complications observed in these patients were: glaucoma secondary, vitreitis, posterior synechiae, cataract, and cystic macular oedema. The posterior synechiae was more frequently in patients with positive IgG response to rHSP60Ye, followed by vitreitis and cataract.

DISCUSSION

The humoral and cellular immune response to human and microbial GroEL-like (HSP60) in the uveitis associated with Behçet’s disease has been widely studied and carefully described.⁶⁻¹² However, the association of HSP60 with pars planitis, VKH, idiopathic AAU, and HLA-B27 associated AAU has not been reported yet.

The objective of this work was to determine the levels of IgG class antibodies to five bacterial HSP60s in the sera of patients with HLA-B27 associated AAU, idiopathic AAU, pars planitis, and VKH. There was a high level of IgG antibodies to rHSP60Ye in the sera of HLA-B27 associated AAU and pars planitis patients and the results of the odds ratio suggested an association between the positive IgG response to rHSP60Ye and the disease. However this high level of antibodies was not found for the other HSPs tested. These results could have been because of the small sample size used in our study; perhaps with a bigger sample size we might

Table 1 Relation between positive IgG response to rHSP60Ye and clinical characteristics of the HLA-B27 associated AAU and pars planitis patients

Clinical characteristics	HLA-B27 associated AAU patients			Pars planitis patients		
	Positive* IgG response to rHSP60Ye	Negative IgG response to rHSP60Ye	Significance	Positive* IgG response to rHSP60Ye	Negative IgG response to rHSP60Ye	Significance
Male:female	5:1	4:1	p=0.88	1:1	1:2	p=0.71
Age of examination, years (mean (SD))	30 (10)	31 (16)	p=0.91	15 (7)	18 (13)	p=0.63
Recurrence (%)	33	40	p=0.82	29	33	p=0.82
Eye complications† (%)	83	20	p=0.03	43	50	p=0.81
Spondyloarthropathy (%)	50	20	p=0.15	ND	ND	ND
Level of inflammation‡ (%)	50	80	p=0.33	29	50	p=0.21

*We considered as a positive response to rHSP60Ye, values A_{492nm}≥0.58.
 †The mean complications presented in all patients were: glaucoma secondary, vitreitis, posterior synechiae, cataract, cystic macular oedema.
 ‡Level of inflammation was determined by clinical examination and the grade of cellularity.
 ND, not determined.

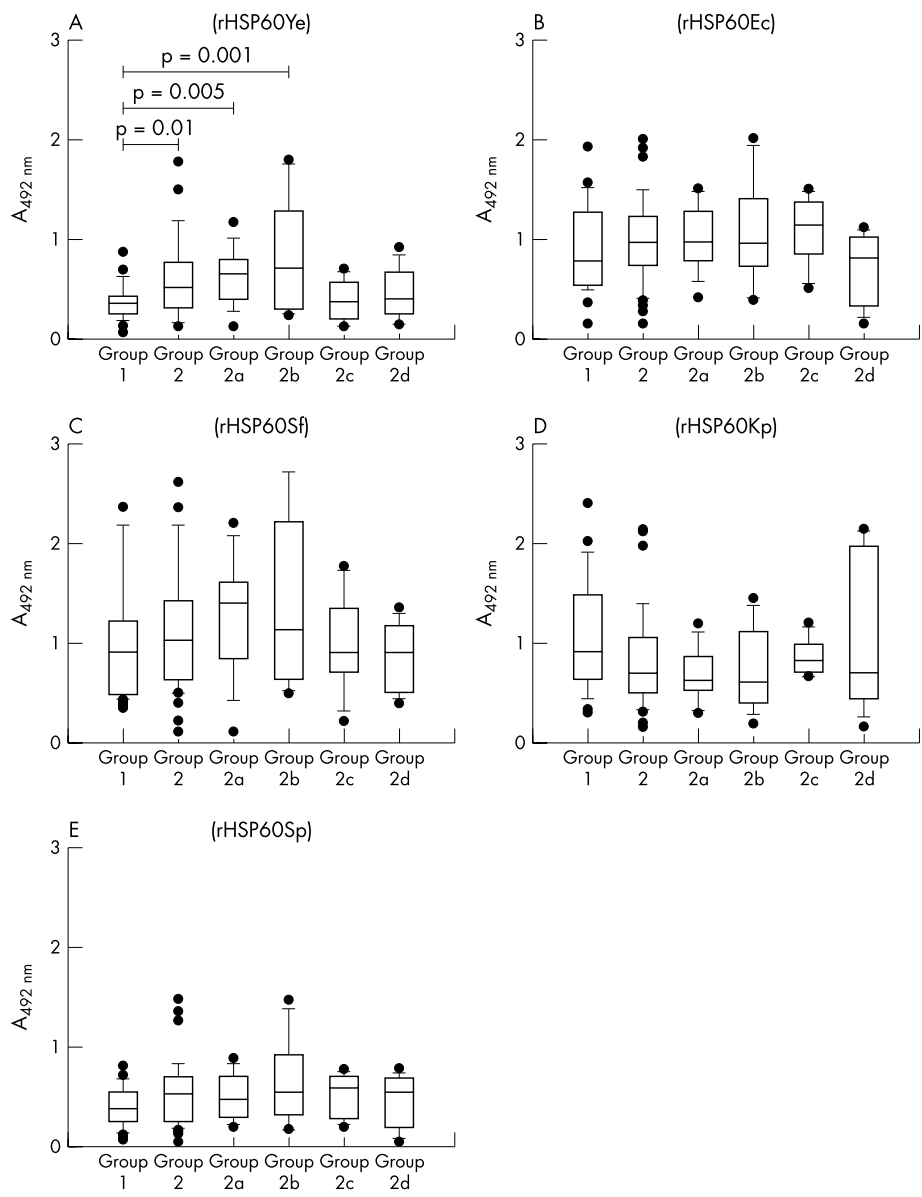


Figure 2 Comparison of bacterial HSP60 IgG antibody levels in uveitis patients and healthy subjects. Group 1, healthy subjects (n=25); group 2, corresponding all the uveitis patients (n=42); group 2a, HLA-B27 associated AAU only with or without spondyloarthropathies (n=11); group 2b, pars planitis only (n=13); group 2c, idiopathic AAU only (n=8), and group 2d, VKH only (n=10). Levels of anti-rHSP60Ye (A), anti-rHSP60Ec (B), anti-rHSP60Sf (C), anti-rHSP60Kp (D), and anti-rHSP60Sp (E) were determined by ELISA. Sera were diluted 1:300 and assayed by triplicate. Each bacterial rHSP60 were used at 0.5 µg/well. The graphic shows the median A_{492nm} value of each group and the percentile at 25 and 75%.

have observed an association with some of the other HSP60s assayed, because of the high degree of homology among HSPs.

Individuals with HLA-B27 are especially at risk of developing ankylosing spondylitis and Reiter's syndrome. Acute anterior uveitis often occurs in association with these diseases or in HLA-B27 positive individuals without joint disease. In our study, we found that the majority of patients with HLA-B27 associated AAU did not present clinical evidence of joint disease. However, *Y enterocolitica* infection in humans causes a broad spectrum of diseases ranging from acute bowel disease to extraintestinal manifestations such as reactive arthritis, erythema nodosum, and uveitis.¹⁵ A prospective study of patients with AAU showed evidence of recent *Yersinia* infection but their clinical course and prognosis did not differ significantly from those encountered in HLA-B27 positive patients.¹⁶ Saari *et al* found that in HLA-B27 positive children, especially after the age of 10 years, the *Yersinia* infection may occasionally trigger reactive iritis or conjunctivitis which often occurred together with other HLA-B27 associated rheumatic diseases.¹⁷ Careless *et al* demonstrated that patients with AAU showed increased frequency

of anti-*Yersinia* antibodies compared with controls, and the majority of these patients were HLA-B27 positive.¹⁸ In this study we obtained similar results, but with rHSP60Ye we found that eye complications were more often in patients with positive IgG response to rHSP60Ye than those with a negative response. These results are in agreement with the findings of Huhtinen *et al*, who reported high levels of IgA class antibodies to chlamydial HSP60 in HLA-B27 positive patients with AAU. They also reported that eye complications were more often evident in patients with positive IgA response to chlamydial HSP60.¹⁹ On the other hand, significantly higher serum IgG antibody levels to HSP60Kp and HSP60Ye in HLA-B27 positive patients with ankylosing spondylitis have recently been reported.^{20, 21}

In a previous study, we found that the most antigenic regions in HSP60kp, HSP60Ec, and HSP60Ye correspond with the amino acid residues 282–290, 360–368, and 390–398, but that HSP60Ye has another added antigen region in the 529–537 residues.²² Therefore, this last epitope of *Y enterocolitica* could be involved in the characteristic response observed in the patients to HSP60Ye. The differential humoral immune response observed between B27 positive and negative

subjects to rHSP60Ye could be caused by T cells from B27 positive subjects, but not B27 negative, recognising specific residues of this protein resulting in the T-B cooperation required for the antibody response and possibly the pathogenic response.²³

We suggest that the HSP60Ye antibodies found in patients with uveitis could have been produced during bacterial infection and these antibodies could trigger an autoimmune reaction through molecular mimicry between the human and microbial HSPs. This mechanism is in agreement with the results of Hu *et al* and Uchio *et al*, who reported that human and microbial conserved peptides of HSP60 can induce uveitis in Lewis rats.^{24 25}

To our knowledge, the association of microbial HSP60 with pars planitis has not been reported yet. In the present study, patients with this disease had a high level of IgG class antibodies to HSP60Ye. de Smet and Ramadan found that human inducible HSP70 could be linked to the aetiology of pars planitis.²⁶ The aetiology of pars planitis is unknown but there is evidence to think that it is an autoimmune disease directed against the vitreous humour.^{27 28}

We suggest that HSP60Ye could be associated in the aetiology of HLA-B27 associated AAU and pars planitis.

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Authors' affiliations

J C Cancino-Díaz, Laboratorio de Inmunología ocular del Instituto de Oftalmología Fundación Conde de Valenciana, Mexico and Departamento de Microbiología, Laboratorio de Microbiología General de la Escuela Nacional de Ciencias Biológicas del Instituto Politécnico Nacional, Mexico

L Vargas-Rodríguez, N Grinberg-Zylberbaum, Departamento de Uvea del Instituto de Oftalmología Fundación Conde de Valenciana, Mexico

M A Reyes-López, Laboratorio de Microbiología Molecular del Instituto de Oftalmología Fundación Conde de Valenciana, Mexico

M L Domínguez-López, A Pablo-Velazquez, M E Cancino-Díaz, Departamento de inmunología, Laboratorio de inmuoquímica I, Escuela Nacional de Ciencias Biológicas del Instituto Politécnico Nacional, Mexico

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