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# Increased titres of IgM anti-heparan sulfate antibody in Behçet's disease

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Received on August 29, 2006; accepted in revised form on September 11, 2006.

Clin Exp Rheumatol 2006; 24 (Suppl. 42): S104-S107.

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**Key words:** Behçet's disease, heparan sulfate, endothelium, antibodies.

## ABSTRACT

**Objective.** Endothelial dysfunction is crucial in Behçet's disease (BD) pathogenesis, and measures of endothelial damage are potential markers of BD activity. Heparan sulfate (HS) is the most abundant proteoglycan in the endothelial cells, and anti-HS antibodies have been reported in subjects with vascular damage, due to vasculitis/vasculopathy. The aim of our study was to measure serum anti-HS antibodies in patients with BD and to determine whether their presence correlates with disease activity or clinical manifestations.

**Methods.** Thirty-two patients with BD (21 men, 11 women) (median age  $36.81 \pm 2.0$  years) were considered. Of these, 13 had clinically active disease at the time of study. The mean disease duration was  $7.31 \pm 8.2$  years (median 6 years). Anti-HS antibodies were measured by ELISA. As controls, sera from 40 sex- and age-matched healthy subjects, and 78 age-matched patients with systemic lupus erythematosus (SLE) were studied.

**Results.** Anti-HS IgM antibody titres were significantly higher in BD patients compared to healthy subjects ( $p=0.016$ ) and SLE controls ( $p=0.0008$ ). No differences in anti-HS IgG antibody titres were observed among the 3 groups. Using categorical data, increased titres of IgM anti-HS antibodies were significantly more frequent in patients with BD vs patients with SLE ( $p=0.02$ ). The presence of the antibodies, of either isotype, did not correlate with disease duration, disease activity or clinical manifestations.

**Conclusions.** BD patients have increased IgM anti-HS antibody titres compared to healthy and SLE controls. These antibodies did not correlate with

disease activity or discrete clinical features, but might be relevant for pathogenic mechanisms of the disease.

## Introduction

Behçet disease (BD) is a multisystemic vasculitis, characterized by recurrent oral and genital ulcerations, uveitis, skin lesions, and thrombosis (1). Lacking pathognomonic signs or laboratory test, the diagnosis of BD still relies on clinical findings, and numerous sets of diagnostic criteria have been proposed (2-4). The etiology is still unknown, but autoimmune, infectious, and genetic factors seem to play a role (5-7). Endothelial dysfunction is crucial for the pathogenesis of vascular damage, and represents a trigger of the pre-thrombotic status of BD (8). Several parameters of endothelial damage have been investigated as potential biochemical markers of BD, including leptin (9), endothelin-1 and nitric oxide (10), interleukin-8 (11), soluble E-selectin (12), lipoprotein (a) and nitrites (13). However, specific and reliable markers of endothelial dysfunction that correlate with clinical activity are still lacking. The mechanisms underlying the vascular endothelial damage are yet to be identified. There is evidence that humoral immunological damage to endothelial cells may play a role. Anti-endothelial cells antibodies (AECA), which are capable of increasing leukocyte adhesiveness, and vascular thrombosis, have been reported in BD (14-16). However, AECA have been inconsistently associated with disease activity or severity (17, 18).

Heparan sulfate (HS) is a glycosaminoglycan, a hexosamine-containing complex polysaccharide, with *o*-glycosidyl linkages to proteins to form proteoglycans. HS is the most abundant proteo-

glycan in endothelial cells, where it plays important roles in anticoagulation, maintenance of vessel barrier, processes of cell adhesion, and control of angiogenesis (19). Antibodies to HS have been described in subjects with vascular injury, due to vasculitis/vasculopathy, suggesting a possible role of the antibodies as a marker of endothelial damage (20).

Considering that HS is highly expressed in the endothelium, and that the endothelium is a target in BD, we hypothesized that antibodies to HS might be associated with BD and reflect disease activity, as was previously proposed for AECA (14-16). Since the presence of anti-HS antibodies has never been investigated in BD, the objective of our study was to measure these antibodies in the sera of patients with BD and to assess whether their presence correlates with disease activity or specific clinical manifestations (eye lesions, muco-cutaneous alterations, joint involvement).

## Materials and methods

Thirty-one consecutive patients with BD, seen at the Departments of Immunology and Ophthalmology of our University, were enrolled in a 6-month period study. Another BD patient, a 33-year-old man affected with iridocyclitis, aphthae and axonal multineuropathy, was recruited from the Department of Neurosciences, for a total of 32 patients (21 men, 11 women) (median age 36.81±12.0 years) with no significant differences in age between the 2 sexes (Mann-Whitney test). The mean duration of disease was 7.31 ± 8.2 years (median 6 years, range: 1 – 18). All the patients fulfilled the International Study Group criteria for the diagnosis of BD (2).

At the time of the study, 13 patients had clinically active disease with at least one major criterion with or without any number of minor criteria. Of these, 12 patients complained of muco-cutaneous manifestations (orogenital ulcerations, erythema nodosum and folliculitis), 6 had ocular lesions (retinal vasculitis and occlusion, vitritis, hyphopion), one had peripheral neurological involvement with severe axonal multi-neuropathy; arthralgia was present in 9

patients. In 9 patients symptoms occurred in more than one system (ocular and muco-cutaneous in 3, ocular and arthralgia in 3, muco-cutaneous and arthralgia in 3). The remaining 19 patients were asymptomatic at the time of evaluation. No patients presented with vascular thrombosis at the time of blood sampling. At the time of the study, 7 patients were free from therapy because they were considered to be in remission, whereas the remaining 25 patients were taking immunosuppressants: 20 patients were on steroids (15 prednisone, 4 methylprednisolone, 1 deflazacort) alone (5 patients) or associated with other drugs (6 azathioprine, 5 cyclosporin A, 1 cyclophosphamide, 1 mycophenolate mofetil, 2 methotrexate). Four patients were in monotherapy with cyclosporin A, mycophenolate mofetil, or chlorambucil; five were also taking colchicine.

The control groups consisted of 40 sex- and age-matched healthy subjects (median age 40.8 ± 9.6 years), and 78 age-matched patients (11 men, 67 women; mean age 37.1 years ± 9.7) with systemic lupus erythematosus (SLE) fulfilling the ACR classification criteria (21). SLE disease activity was measured with the European Consensus Lupus Activity Measurement (ECLAM) score (22). Fasting blood was collected from each participant by standard venipuncture into evacuated tubes without EDTA. Serum was isolated and stored at -20°C until analyzed.

All investigations were performed after obtaining informed consent from subjects.

## Enzyme-linked immunosorbent assay (ELISA) for the detection of anti-HS antibodies

Anti-HS antibodies were measured by ELISA, using biotinylated antigens and avidin-coated microwells, as previously described (20). Briefly, HS from porcine intestinal mucosa (SIGMA Immunochemical, St. Louis, MO) was biotinylated using biotin hydrazide and EDC as crosslinker, and applied (1 µg/well) to avidin-precoated microwells. After saturation with 1% BSA in PBS, increasing dilutions of patients' sera were added and incubated over-

night at 4°C, followed by peroxidase-conjugated goat anti-human IgM or IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) for 2 hours. Wells coated only with avidin-BSA served as controls for each dilution. After washing, 100 µL of developing solution containing 0.05 M Na<sub>2</sub>HPO<sub>4</sub>, 0.024 M sodium citrate pH 5, 0.08 % o-phenylenediamine, and 0.08% H<sub>2</sub>O<sub>2</sub> were added. Reaction products were measured spectrophotometrically at 450 nm in a Biotek EIA reader. The titre for each specimen was taken as the highest dilution at which the optical density (OD) reading was 0.1 units greater than in the corresponding avidin-BSA coated wells. As positive control for the assay, mouse monoclonal anti-HS Antibodies were used (SIGMA). Titres ≥ 51200 for IgM and 25600 for IgG were considered positive, as in our previous studies (20, 23).

## Absorption studies

The specificity of antibodies to HS was assessed by absorbing the patients' serum with 100 µg of HS antigen bound to avidin acrylic beads (SIGMA), as previously described (20). After an overnight incubation at 4°C, the samples were centrifuged and the supernatant tested for anti-HS activity by ELISA. As control, sera were absorbed with biotin bound to avidin acrylic beads.

## Statistical analysis

The Student t-test was used for normally distributed variables, while the Mann-Whitney U Test and the Kruskal-Wallis were performed for ordinal variables. The Chi Square Test was used for categorical variables. To test the linear correlation between two variables the Spearman's Rho was performed. The significance level was set at p < 0.05. All data were analyzed using the STATISTICA 6.0 software package for Windows (StatSoft, Tulsa, OK, USA).

## Results

Anti-HS IgM antibody titres were significantly higher in BD patients when compared to healthy subjects (Mann-Whitney U test: p = 0.016) or SLE controls (Mann-Whitney U test: p = 0.0008), while no difference in anti-HS IgM

antibody titres was observed between healthy subjects and SLE patients (Mann-Whitney U test:  $p = 0.36$ ) (Fig. 1). Anti-HS IgG antibody titres were similar in BD, healthy subjects and SLE (Kruskal-Wallis test:  $p = 0.99$ ).

Using categorical data, increased titres of IgM anti-HS antibody were present in 5 of the 32 (15.63%) patients with BD vs 2 of the 78 (2.56%) patients with SLE (Fisher Exact Test:  $p = 0.02$ ).

Increased titres of IgG antibodies to HS were present in 2 patients with BD (6.25%) vs 7 in the SLE group (8.97%). No correlation was observed between IgM or IgG-anti-HS antibodies and any clinical feature or disease activity in BD and SLE patients.

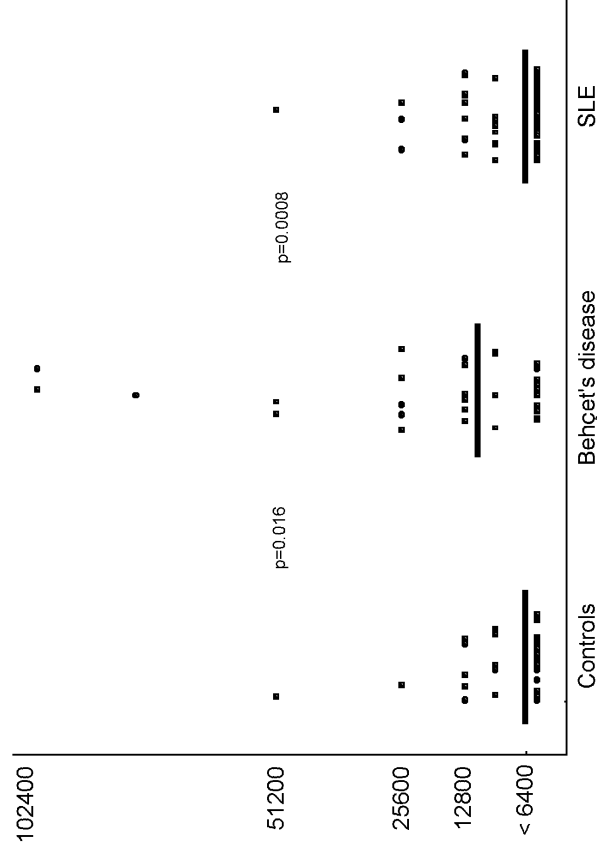
The mean duration of the disease was significantly higher in the SLE group ( $10.7 \pm 3.9$  years) than in BD group ( $7.3 \pm 8.2$  years) (Mann-Whitney U test:  $p < 0.00001$ ). No correlation was observed between IgM or IgG anti-HS antibodies and disease duration in patients with BD (Spearman Rho = 0.24 and 0.10, respectively).

Absorption of the sera from anti-HS antibody positive patients with HS eliminated the reactivity against HS, whereas preabsorbing the serum with biotin did not reduce the reactivity, thus supporting the antibody specificity.

## Discussion

In the present study we demonstrated a significantly increase of IgM anti-HS antibody titres in BD patients over healthy subjects and SLE controls. To the best of our knowledge, this is the first investigation to report high IgM anti-HS antibodies in BD.

Looking for markers of BD activity, we focused on HS, which is the most abundant proteoglycan in vessels and a potential target in endothelial dysfunction. Previous studies showed increased anti-HS antibodies in inflammatory and vasculitic diseases (20). Endothelial HS proteoglycans are susceptible to cleavage by polymorphonuclear neutrophils (24), a major histopathological finding of active BD lesions (25), where they play an important pathogenic role. Polymorphonuclear neutrophils also express multiple heparanases



**Fig. 1.** IgM anti-HS antibody titres in healthy subjects (controls), patients with Behcet's disease and systemic lupus erythematosus (SLE). Anti-HS IgM antibody titres were significantly higher in BD patients when compared to healthy subjects ( $p = 0.016$ ) or SLE controls ( $p = 0.0008$ )

(26), endoglycosidases that degrade HS especially in response to inflammatory stimuli, thus activating endothelial cells, facilitating cellular extravasation as well as vascular damage (27). In line with the data on AECA as markers of BD activity (14–16), we aimed at evaluating whether other antibodies targeting endothelial molecules could be informative as markers of disease activity. In our study, IgM anti-HS antibodies did not correlate with disease activity or discrete clinical features. However, it should be noted that reliable measures of BD activity are still lacking.

Our findings support the hypothesis that the endothelium might be the target of a humoral autoimmune phenomenon in BD, with the IgM isotype being involved. Circulating antibodies to microvascular endothelial cells have been reported in BD (28). It is noteworthy that only the IgM antibodies showed reactivity towards the human dermal microvascular endothelial cells and reacted on Western blots with a 44-50-kd surface antigen, recently identified as alpha-enolase (29), a glycolytic enzyme involved in autoimmune and systemic diseases (30).

The mechanisms responsible for generation of antibodies to HS are unclear. Glycosaminoglycans are normally poor-

ly immunogenic, but modifications in the structure of glycosaminoglycans, which are likely to occur in an inflammatory environment, may induce conformational changes that make them immunogenic. Glycosaminoglycans have been proposed as target antigens in autoimmunity (31), and alterations of glycosaminoglycans or their functions have been described in both rheumatological and immune-mediated pathological conditions (32, 33).

IgM anti-HS antibody titres in SLE patients were significantly lower than those found in BD and similar to those found in healthy subjects. Anti-HS antibodies have been reported in SLE patients in association with renal involvement (34, 35), consistent with the fact that HS is the major proteoglycan in the glomerular basement membrane (36). A cross-reactivity between anti-DNA antibodies and proteoglycans has also been observed (37), and a SLE-like disease was induced experimentally following immunization with HS (38). The lack of anti-HS antibodies in our SLE population may be due to the fact that the patients were recruited from a Division of Rheumatology, and patients with severe renal involvement were likely under-represented.

It is not known whether anti-HS antibodies arise secondarily to endothelial



damage mediated by different causes (neutrophils, oxidative stress, etc.), but they might contribute to vascular damage and breakdown of the endothelial integrity, as it has been suggested for AECA (39). The different antigenic profile of endothelial cells in different tissues might explain the heterogeneity of the clinical manifestations and correlations with antibody findings. A study on a larger group of BD patients with vascular complications might help to better define the role of the anti-HS antibodies as a possible marker of endothelial damage.

In conclusion, BD patients have increased IgM anti-HS antibody titres which might be relevant for pathogenic mechanisms of the disease.

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