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Heat Shock Proteins in Behçet Syndrome

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Behçet syndrome (BS) is a systemic vasculitis of unknown etiology that affects the skin, mucosa, joints, eyes, central nervous system, gastrointestinal system, arteries, and veins. It is generally believed to have a complex genetic background where both innate and adaptive immune systems are activated through environmental factors, such as infections, and auto-antigens. Heat shock proteins (HSPs) are highly conserved and immunogenic endogenous proteins that are thought to play both an enhancing and regulating role in several autoimmune and inflammatory diseases, such as rheumatoid arthritis, juvenile idiopathic arthritis, and Type I diabetes. There is evidence supporting the role of various microorganisms in BS, which may be using a common pathway to trigger or activate BS through molecular mimicry. The significant homology between microbial and human HSPs suggests that HSPs could serve as a common trigger. This review summarizes the work on the role of HSPs in the pathogenesis of BS. However, it remains unknown whether the HSPs detected in BS lesions play a causative role, their presence is a result of the ongoing inflammation, or they have a protective role against inflammation, as suggested in some other diseases.

Behçet syndrome (BS) is a systemic vasculitis with an unknown etiology. The main disease domains are skin-mucosa involvement with recurrent oral ulcers, genital ulcers, nodular and papulopustular lesions, musculoskeletal involvement, eye involvement, usually in the form of posterior or panuveitis, vascular involvement with venous and arterial thrombosis and arterial aneurysms, central nervous system involvement, and gastrointestinal involvement. The pathergy phenomenon, a hyperreactivity to trauma, has high specificity for BS and may be used as a diagnostic test. The diagnosis is usually made during the third or fourth decade of life. BS runs a more severe course with more major organ involvement, causing irreversible damage among men, especially among those who are younger at disease onset.[1]

The current view is that BS has a complex genetic background where both innate and adaptive immune systems are activated through environmental factors, including infections and auto-antigens.[2] Carrying an HLA-B51/B5 allele is associated with a 5.8-times higher risk of having BS.[3]

Papulopustular lesion and arthritis more frequently occur together and this association is more common among familial BS patients, and frequently shared by both members of the same family.[4-6] Enthesopathy was also frequent in BS patients with the papulopustular lesion and arthritis association, without sacroiliitis or HLA-B27 positivity.[7,8] Together with the observation that the pustular lesions of BS are infected with several microorganisms, including staphylococci, streptococci, and Prevotella species, it was proposed that the papulopustular lesion, arthritis, and enthesopathy association may have a similar pathogenesis to acne associated arthritis, involving an infectious etiology.[9]

THE ROLE OF INFECTIONS IN THE PATHOGENESIS OF BEHÇET SYNDROME

There are several clues from clinical studies, other than the acne and arthritis association, suggesting an infectious etiology of BS, such as a higher rate of tonsillectomy, cold sores, and dental caries, a higher number of siblings, a late birth order, an earlier age at first sexual intercourse, and a history of travel to countries with a high incidence of BS.[10] Moreover, it was shown that the frequency of a positive pathergy reaction decreased with surgical cleaning of the skin before the procedure.[11] The efficacy of penicillin on mucocutaneous and joint lesions of BS also supported this theory.[12,13]

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Oral ulcers are present in almost all patients with BS, and they are usually the first manifestation of BS, starting several years before the other manifestations. Thus, oral microbiota has been proposed to play an important role in the pathogenesis of BS.[14] Streptococci, which are generally related to dental infections, are the prominent bacteria in the oral flora. Biopsies of oral ulcers showed components of *S. sanguinis*[15] and serum antibody titers to certain S. sanguinis strains were higher in BS patients compared with healthy controls.[16] Although no specific microorganisms have been identified to directly affect BS pathogenesis, an association has been suggested with increased *Streptococcus salivarius*, *Streptococcus sanguinis*, *Rothia denticariosa*, *Haemophilus parainfluenzae*, *Bifidobacterium*, *Prevotella*, and *Scardovia* and lower colonization of *Neisseria* spp, *Veillonella* spp, *Alloprevotella rava*, and *Leptotrichia* spp.[17-19]

Moreover, streptococcal skin tests caused exacerbations of BS manifestations in patients. Biopsy samples from various skin lesions of BS patients showed the Bes-1 DNA, which encodes peptides 229-243 and 373-385 of *S. sanguinis* and was expressed in the cytoplasm of monocytes, infiltrating the vascular wall of these lesions.[20] It was later shown that the 373-385 peptide of Bes-1 stimulated interferon-gamma (IFN-gamma) and IL12 production in peripheral blood mononuclear cells of BS patients.[21]

A hypersensitivity to streptococci among BS patients was proposed and T-cell responses were studied to explain this hypersensitivity. It was shown that peripheral blood CD8+γδ+ T-cells showed a significantly proliferative response to *S. sanguinis* strain KTH-1.[22] T-cells stimulated with KTH-1 produced larger amounts of IL6 and IFN-gamma in BS patients.[23]

Although streptococci have been studied most extensively, there is evidence to suggest that infections with other bacteria and even viruses, including HSV, may be associated with BS. It was shown that *S. sanguinis* strain KTH-1 antigens, as well as E. coli derived antigens and staphylococcal superantigens SEB and SEC1 cause increased IFN-gamma production by T-cells in BS patients.[24] Altogether, it seems more likely that several microorganisms may be using a common pathway to trigger a series of events. The significant homology between microbial and human heat shock proteins (HSPs) led to the idea that HSPs could be such a common trigger through cross-reactivity between the human and bacterial HSPs.

HEAT SHOCK PROTEINS AND AUTOIMMUNITY

Heat shock proteins (HSPs) are highly conserved molecules whose expression levels are increased under cellular stress to maintain cellular homeostasis. This was first reported in temperature-shocked Drosophila by Ritossa[25] in 1962 as the puffing pattern of the chromosome, which indicates chromosome activity. During subsequent years, similar chromosomal changes have been observed in both prokaryotic and eukaryotic organisms not only with temperature shocks but also under certain cellular stress conditions, such as oxidative stress, infection, and inflammation.[26] HSPs are classified according to their molecular weight into large HSP families (including HSP40, HSP60, and HSP90) and small HSP families (including HSP22, HSP27, and HSP32). They have different cellular localizations and show various functions in response to cellular stress.[27,28]

HSPs, which are also called stress proteins, play a role in the proper folding of newly synthesized and misfolding proteins and in the degradation of damaged proteins. In addition to their chaperone function, they have pro- and anti-inflammatory roles, as well as a role in antigen presentation.[29] HSPs are usually present at sites of inflammation. HSPs, such as HSP40, HSP60, HSP70, and HSP90, are suggested to play a role in the pathogenesis of several autoimmune and inflammatory diseases, such as rheumatoid arthritis, juvenile idiopathic arthritis, and Type I diabetes. The current concept is that HSPs not only enhance but also regulate autoimmunity. Studies have shown that in addition to mediating tissue inflammation and organ pathology, they also protect against and help suppress inflammation. Such a regulatory role has been suggested to be operative in Kawasaki disease, in the adjuvant arthritis model induced in Lewis rats by heat-killed Mycobacterium tuberculosis, and in patients with juvenile idiopathic arthritis. Moreover, protection against experimental Sjogren's syndrome was suggested with the pretreatment of NOD mice with mammalian Hsp65.[30] The mechanisms that are proposed to explain the initiation and propagation of immune pathology by HSPs are activation of innate immunity, stress induced HSP expression causing altered antigen processing and presentation, and molecular mimicry. Due to their highly conserved nature, microbial and human HSPs show significant homology.[31] Thus, the T-cells and antibodies induced by microbial HSPs may target homologous self HSPs and cause tissue damage through molecular mimicry. Considering the clinical clues to an infectious etiology in BS, the specific oral flora and microbial growth in papulopustular lesions and the data on streptococcal hypersensitivity, this scenario of molecular mimicry fits well in BS pathogenesis.

HEAT SHOCK PROTEINS IN BEHÇET SYNDROME

Evidence for the presence of several different microorganisms, including various species of streptococci, brought the idea that a common antigen or antigens may be playing a role in the pathogenesis of BS.[32] HSP seemed to be a good candidate since HSPs of several microorganisms and human HSPs were largely homologous.

The first study seeking a relationship between HSP and BS was by Lehner et al.[33] They aimed to see whether monoclonal antibodies to the 65 kDa HSP (HSP65) react with certain streptococcal species which were formerly implicated to play role in the pathogenesis of BS, and they identified cross-reactive anti-mycobacterial HSP65 with oral mucosal homogenates and streptococci species that are frequently present in the oral flora.

They also examined the sera of BS patients and healthy controls for antibodies to streptococci and to the 65 kDa recombinant stress proteins and 64 kDa proteins isolated from *E. coli* and *M. bovis*. A significant increase in the level of IgA antibodies against mycobacterial HSP65 was observed. Moreover, antibodies against

S. sanguinis and S. pyogenes were detected at 65 kDa bands, similar to responses against mycobacterial HSP65 of oral mucosal extracts. Considering that human 60 kDa HSP (HSP60) shows about 60% homology with mycobacterial and streptococcal 65 kDa HSP (HSP65), this study provided the first evidence for molecular mimicry through HSPs in BS.

Eye Involvement

Pervin et al.[34] identified four peptides (111-125, 154-172, 219-233 and 311-325) from the mycobacterial HSP65 and their homologous human 60 kDa HSP (HSP60) peptides (136-150, 179-197, 224-258, 336-351) with T-cell epitope mapping. These peptides were shown to stimulate the proliferation of γδ+ T-cells in BS patients.[34] It was observed that this response was not restricted to HLA-B51, and patients with eye and joint involvement had higher response rates than BS patients with only mucocutaneous involvement. Especially the peptides 111-125 and 311-326 and their human homologous counterparts 136-150 and 336-351 stimulated the highest lymphocyte responses in BS patients with eye involvement.

Direskeneli et al.[35] mapped the B-cell epitopes of the mycobacterial HSP65 in BS patients. They observed significantly higher IgA and IgG responses to mycobacterial peptides 111-125, 154-172, 311-326 and human peptides 136-150, and 336-351 in BS patients compared to controls. Sequential analyses suggested that these IgA and IgG titers might increase during uveitis attacks.

T-cell responses to the previously mentioned peptides were studied among Japanese and Turkish BS patients as well.[36,37] Kaneko et al.[36] confirmed the role of HSP60 peptide 336-351 in causing proliferation of T-cells in Japanese BS patients. This proliferation was not present in their healthy controls or rheumatoid arthritis patients. Moreover, they also showed that this proliferation was associated with the presence of ocular but not other manifestations of BS.

Among the 28 BS patients that they had included in their study, 18 had uveitis and 10 had other manifestations. The HSP60 peptide 336-351 showed a significantly higher proliferative response in T-cells of BS patients with ocular lesions compared to those who did not have ocular lesions.

The other 3 peptides that had showed proliferative responses in British BS patients (136-150, 179-197 and 244-258) also provided significantly higher proliferation of T-cells in BS patients compared to the control mycobacterial HSP peptide 91-105. However, this difference was not statistically significant when compared to T-cells from healthy controls.

Furthermore, Kaneko et al.[14] tried to find out which T-cell subsets are proliferated most by the peptide 336-351 and showed that only CD4+ T-cells were proliferated.

Direskeneli et al.[37] studied the peripheral blood responses to the same mycobacterial and homologous human HSP peptides in Turkish BS patients. They included 49 patients with BS, 46 patients with various inflammatory diseases such as rheumatoid arthritis, idiopathic uveitis, and recurrent oral ulceration, and 34 healthy controls. Among the BS patients, 12/23 (52%) responded to at least one of the mycobacterial peptides, compared to 3/18 (17%) of diseased controls and none of the healthy controls ($p = 0.002$). Similarly, 13/23 (57%) BS patients responded to at least one of the homologous human peptides compared to 2/18 (11%) diseased controls and none of the healthy controls ($p < 0.001$). They further studied the responses to mixtures of the 4 mycobacterial and 4 human peptides and observed that the mean stimulation index that they calculated as the cpm of cells with antigen divided by the cpm of cells without antigen, was significantly higher among BS patients compared to both diseased and healthy controls, for both mixtures. The frequency of a positive response was 15/26 (58%) among BS patients, compared to 5/28 (18%) among diseased controls ($p = 0.001$) whereas no positive responses were observed among healthy controls. For the human peptides, 10/26 (38%) of BS patients had a positive response compared to 2/28 (7%) of diseased and none of the healthy controls ($p < 0.01$). However, there was no significant difference between active and inactive BS patients regarding T-cell responses to mycobacterial or human peptide mixtures in their study.

Hasan et al.[38] showed that γδ+ T-cell preferentially responded to these peptides, especially in active patients. They further suggested that this specific proliferation of γδ+ T-cells by the 4 peptides could be used as a diagnostic test for BS. They showed that 25/33 (76%) BS patients showed significant γδ+ T-cell responses to the mycobacterial peptides compared to 2/55 (3.6%) of diseased and healthy controls. T-cell responses were correlated with disease activity and the sensitivity of the test increased when active patients were included. The stimulation indices of homologous human peptides were lower than the mycobacterial peptides.

Stanford et al.[39] injected these peptides into Lewis rats and examined these rats for the development of uveitis and other manifestations of BS to find out whether these peptides have a role in the pathogenesis of BS. They used the four synthetic peptides derived from the sequence of HSP65 which had stimulated T-cells of BS patients in their previous study (111-125, 154-172, 219-233, 311-326) as well as a control peptide (91-105) which did not stimulate T-cells of BS patients. The human homologous HSP peptides that they used were 136-150, 179-197, 224-258, 336-351 and 116-130 for control. After injecting these peptides into Lewis rats, they followed the rats from day 7 to week 4 for the development of uveitis. Uveitis was induced in 7/10 rats injected with 111-125, 3/10 rats injected with 311-329 and among the human homologous peptides, in 7/11 of rats injected with 136-150 and 11/12 of rats injected with peptide 336-351. The control mycobacterial peptide 91-105 and human peptide 116-130 did not induce uveitis in any of the 13 rats into which they were injected. The development of uveitis usually started during the third week and lasted for 2-4 days. Clinical manifestations of rats injected with each of the peptides and mycobacterial and homologous human peptides were similar. There were no other manifestations of BS such as oral or genital ulcers or other skin lesions.

The same group extended their work by measuring the level of IgG and IgA antibodies, along with clinical and histological evaluation of uveitis, after injecting Lewis rats with the same mycobacterial and human peptides.[40] This time, 14/20 rats injected with the mycobacterial peptides 111-125, 9/20 injected with the peptide 311-326, 15/21 rats injected with human homologous peptide 136-150 and 19/22 injected with the peptide 336-351 developed uveitis both clinically and histologically. Clinical examination showed inflammatory cells in the anterior chamber, enlargement, and tortuosity of the iris vessels and posterior synechia. Histological examination showed that mononuclear cell infiltration was prominent in the anterior chamber, iris, and ciliary body. When IgG and IgA antibodies were measured with ELISA, it was observed that rats that developed uveitis had significantly higher serum IgG levels for mycobacterial peptide 311-326 and its homologous human peptide 336-351, and higher serum IgA levels to peptides 111-125, 311-326 and 336-351, compared to rats without uveitis. The level of IgG antibodies was higher than IgA, but the IgA level increased earlier than IgG levels. It was suggested that since certain peptides within HSP60 may function as T-cell epitopes and induce uveitis, they may play a role in the pathogenesis of BS through a cross-reactive immune response caused by T-cells shared by human and microbial HSPs.

However, it should be noted here that the uveitis observed in these experiments is somewhat different from the uveitis in humans with BS, which is usually characterized by posterior segment findings such as vitreal inflammatory changes, and retinal involvement along with anterior chamber findings.[41] Interestingly, in a recent study, no response to HSP60 was observed in any T-cell lines derived from the intra-ocular fluid of BS patients with uveitis, whereas non-peptide prenyl pyrophosphate reactive γδ+ T-cells were present.[42] Moreover, no other manifestations of BS developed in the rats injected with mycobacterial and human homologous peptides.

In previous studies, experimental uveitis was induced in Lewis rats through immunization with retinal soluble antigen or interphotoreceptor binding protein.[43,44] Moreover, when oral or nasal tolerization with the retinal soluble antigen or its peptide was performed, the development of uveitis by immunization with the retinal soluble antigen was prevented.[45,46]

However, when the same tolerization was attempted by repeated oral or nasal administration of peptide 336-351 against HSP derived peptide induced uveitis, the prevention of uveitis was not successful. Moreover, uveitis developed after mucosal immunization with the peptide through both oral and nasal induction. Hu et al.[47] showed that oral or nasally administered peptide 336-351 induced uveitis. The uveitis that was induced orally and/or nasally was clinically and histologically similar to the uveitis that was induced by subcutaneous injection of peptide 336-351 showing iridocyclitis, accumulation of inflammatory cells in the anterior chamber and loss of photoreceptors. There was no mononuclear cell infiltration in the posterior segment. It was suggested that this may be quite similar to the clinical situation in BS, HSPs of oral microorganisms such as streptococci may cause immune responses through cross-reaction with mucosal HSP and play a role in the development of uveitis. After the failure to tolerize against uveitis in Lewis rats by oral and nasal administration of peptide 336-351, it was attempted to use recombinant cholera toxin B subunit (rCTB) by covalently linking it to peptide 336-351 derived from human HSP.[48] It was previously shown that linking myelin basic protein to rCTB enhances oral tolerance and prevents the development of experimental autoimmune encephalomyelitis.[49,50] Based on this experience, the peptide 336-351 - rCTB conjugate was given to rats orally, and it was observed that the development of uveitis was significantly less compared to oral administration of the peptide 336-351 by itself.[48] In this study, it was observed that significantly more CD4+CD45RClow RT6+ subset of Th2 memory cells were present in the spleens and lymph nodes of tolerized rats with no uveitis compared to immunized rats with uveitis ($p < 0.05$ and $p < 0.005$ respectively). This is similar to what was described in relation to the inhibition of autoimmune thyroiditis and diabetes.[51] In situ hybridization of lymph nodes and the uveal tract showed that L10 and TNF-β mRNA were significantly higher and IFN-gamma and IL12 mRNA were significantly lower in tolerized rats. The authors suggested that the tolerization which protected against uveitis may be associated with a shift from Th1 to Th2 and Th3 cytokines and a regulatory subset of memory cells. They also proposed that mucosally induced uveitis can be prevented by orally administering peptide - rCTB conjugate in BS patients.

The same group then went on to use the peptide 336-351 - rCTB subunit conjugate in BS patients with uveitis.[52] The peptide 336-351 - rCTB subunit conjugate was administered orally 3 times per week to 8 patients with BS in a phase I/II clinical trial. All patients were being treated with immunosuppressives and/or prednisolone for panuveitis, had no ocular attacks for at least 3 months, and had a history of relapse on attempting to reduce immunosuppressives during the previous 6 months. After 3 weeks of treatment with the peptide - rCTB conjugate, the immunosuppressives of the patients were started to be withdrawn. It was possible to withdraw all immunosuppressives in 5 of the 8 patients without any uveitis attacks. In the second phase of the trial, the peptide - rCTB conjugate was discontinued and 2 of the 5 patients experienced uveitis attacks within 1 month. The remaining 3 patients were attack-free for 10 to 18 months of follow-up. No adverse events were reported during the trial.

The response to this agent was associated with a lack of peptide specific CD+ T-cell proliferation, a decreased expression of in Th1 type cells CCR5 and CXCR3, decreased IFN-gamma and TNF-alpha production and decreased C-C chemokine receptor type 7 positive (CCR7+) T-cells and costimulatory molecules CD40 and CD28. Despite the promising results obtained in this phase I/II trial, further controlled trials with this agent, including larger numbers of patients were not conducted.

A possible association between BS uveitis and HSP70 was also suggested. Smet and Ramadan compared serum HSP70 levels of 18 uveitis patients with BS, 15 with sarcoidosis, 13 with pars planitis, 10 with Vogt-Koyanagi-Harada (VKH), and 14 healthy controls. Serum HSP70 levels were significantly higher among

BS, sarcoidosis, and pars planitis patients compared with healthy controls. No increase was observed in VKH patients. On the other hand, there was no correlation between HSP70 levels, disease activity and treatment.[53] Sahebari et al.[54] compared HSP70 serum levels of 26 BS patients with uveitis, 27 BS patients without uveitis, and 25 patients with idiopathic uveitis. HSP70 levels were higher in BS patients with uveitis compared to BS patients without uveitis and idiopathic uveitis. On the other hand, there were no differences regarding serum anti-HSP70 levels between the groups.[54] In contrast to these two studies, in a study where mRNA expression levels of IL18, IFNG, IFNGR, CRP, IL10, and HSP70 were compared between 40 BS patients with uveitis and 30 healthy controls, there was no difference in IL10 and HSP70 mRNA expression levels, despite significantly higher IL18, IFNG, IFNGR, and CRP in active BS patients with uveitis.[55]

The study by Kaburaki et al.[56] identified two peptides, HSP65PD and B51PD derived from HSP65 and HLA-B*51:01, which show high affinity to HLA-B*51:01 which is one of the BS associated HLA alleles. The HSP65PD and B51PD stimulated peripheral blood lymphocytes (PBL) isolated from 47 BS patients with uveitis showed higher proliferation compared to PBL isolated from 19 sarcoidosis patients, 17 VKH patients, 15 patients with the systemic inflammatory disease without uveitis and systemic scleroderma, and 17 healthy controls. These two peptides increase T-cell response in the HLA-B*51:01-positive patients with BS more than in the HLA- B*51:01-negative patients, however, significant differences were not observed between HLA-A*26 -positive and -negative BS patients.[56]

Mucocutaneous Involvement

There are a few studies investigating the local expression of HSP60 in various lesions and involved tissues or organs of BS patients. Local expression of HSP60 in mucocutaneous lesions of BS patients was studied by Ergun et al.,[57] They also studied the amount of local and peripheral blood T-cell receptor (TCR) γδ+ cells which were previously shown to respond to HSP peptides. Flow cytometric analysis of the peripheral blood of 16 BS patients and a total of 18 diseased and healthy controls showed that the mean TCR γδ+ cell counts were similar in BS patients and controls (6.3 ± 4.4 vs. 5.3 ± 3.7). On the other hand, skin biopsies showed increased expression of HSP 60/65 and γδ+ T-cell counts in BS patients. Punch biopsies of 5 positive skin pathergy lesions, 1 genital ulcer, 7 erythema nodosa and 7 papulopustular lesions of BS patients and 2 contact dermatitis, 1 psoriasis, 1 parapsoriasis and 1 cutaneous cell lymphoma biopsies of control patients were assayed for HSP 60/65 (ML-30) staining, and the density of staining was scored in a semi-quantitative way. Among BS lesions 12/21 (57%) were stained (+++), 7/21 (33%) were stained (++) and 1/21 (5%) were stained (+) whereas among the controls 5/17 (27%) were stained (+++), 5/17 (27%) were stained (++) and 7/17 (39%) were stained (+). Both the intensity and total scores were significantly higher in BS lesions ($p = 0.017$ and $p = 0.03$ respectively). Biopsies of 1 genital ulcer, 1 papulopustular lesion, 1 erythema nodosum-like lesion, 3 positive skin pathergy lesions and the same control lesions were scored for γδ+ staining. All BS lesions had moderate to strong staining whereas controls had either negative or less than 15% staining.

In another study looking at the local expression of HSP60 in mucocutaneous lesions of BS, HSP60 expression was investigated in the oral mucosa.[58] Biopsies of 11 oral ulcers of BS patients, 11 oral ulcers of recurrent aphthous stomatitis patients, 11 oral lichen planus lesions and 11 oral mucosa biopsies of healthy individuals were evaluated for HSP expression in the basal, suprabasal and superficial layers of the stratified squamous epithelium, vascular endothelial cells, and infiltrating cells at the lamina propria. HSP60 expression scores were higher in all layers of oral ulcers of BS patients compared to oral mucosa biopsies of healthy controls. On the other hand, HSP60 expression was similar among BS oral ulcers, recurrent aphthous stomatitis lesions, and oral lichen planus lesions. The only difference in HSP60 expression between BS oral ulcers and oral lichen planus lesions was at the suprabasal layer, where HSP expression was higher in BS group compared to oral lichen planus.

Whether the increased HSP expression in the skin lesions of BS patients indicates a role of HSP in the pathogenesis of these lesions or whether their expression is a response to the lesions themselves is not known. Ergun et al.[57] had also rightly pointed to the possibility of their findings being an epiphenomenon, increased HSP expression secondary to the inflammation. This is supported by the presence of some HSP expression in their controls with recurrent oral ulcers as well. Similarly, Deniz et al.[58] observed similar levels of HSP60 expression in BS oral ulcers, recurrent oral ulcers, and biopsy specimens of oral lichen patients.

The concentrations of HSP60 antibody in saliva samples of 65 BS patients were measured by ELISA and compared among patients with mild, moderate or severe disease. The highest HSP60 concentration was found in BS patients with moderate severity. HSP60 concentration was decreased in BS patients using colchicine, and it was speculated that this might be the reason for the lower HSP60 level in patients with severe disease who had used colchicine for a longer duration and a higher dose.[59]

Nervous System Involvement

In a further attempt to understand the role of HSP in the local immune response in BS, Taşçi et al.[60] investigated the humoral immune response to mycobacterial HSP65 in the cerebrospinal fluid (CSF) of BS patients with nervous system involvement. They looked for anti-mycobacterial HSP65 IgG, IgM, and IgA antibodies in the sera and cerebrospinal fluid of BS patients with nervous system involvement. They included 25 BS patients with parenchymal nervous system involvement, 7 BS patients with intracranial hypertension due to dural sinus thrombosis, 8 BS patients without nervous system involvement, 30 multiple sclerosis patients, and 24 patients with non-inflammatory neurological disorders as controls. They observed that the number of patients with IgG, IgM, or IgA anti-mycobacterial HSP60 antibodies in CSF were significantly higher among BS patients with parenchymal nervous system involvement compared to multiple sclerosis and non-inflammatory patients (12/25, 3/30, and 3/24 respectively,

$p < 0.01$). Among the 12 BS patients with parenchymal nervous system involvement, who had positive antibody results, 7 were during an acute attack at the time of CSF and blood collection, while the other 5 had a chronic progressive course. The mean ELISA IgG level in CSF was also higher among BS patients with parenchymal nervous system involvement compared to noninflammatory patients (1.3 ± 0.9 vs. 0.8 ± 0.4, $p < 0.01$).

Another HSP, αB-crystallin was also studied in the sera and CSF of BS patients with nervous system involvement, together with multiple sclerosis patients, Guillain-Barre syndrome patients and patients with non-inflammatory neurologic diseases.[61] It was shown that serum and CSF IgG antibody responses to αB-crystallin were significantly higher in BS patients with nervous system involvement (1.29 ± 0.49) compared to the controls ($p = 0.01$). Serum IgM antibody level was higher in BS patients with neurological involvement (1.83 ± 0.72) and in multiple sclerosis patients (1.57 ± 1.07) compared to other groups ($p = 0.0005$ and $p = 0.046$ respectively). CSF IgM levels were significantly higher in Guillain-Barre syndrome patients (2.09 ± 1.09, $p = 0.007$). The increased humoral responses against αB-crystallin observed in BS patients with neurological involvement, multiple sclerosis patients and Guillain-Barre syndrome patients compared to non-inflammatory neurologic patients may be interpreted as supporting the role of αB-crystallin in the pathogenesis of these inflammatory conditions. Similar to what was observed with HSP60, BS patients with parenchymal nervous system involvement had higher cerebrospinal fluid IgG responses to αB-crystallin compared to BS patients with vascular neurologic involvement.

Lule et al.[62] suggested that neurofilament medium (NF-M) might have a role in the pathogenesis of BS. They observed a similarity between NF-M and amino acids 111-126, 213-232 and 304-363 of mycobacterial HSP65, which were previously shown to cause increased lymphocyte proliferation in BS patients. Immunoreactivity was detected in mouse brain sections incubated with serum samples from 34 BS, 12 systemic lupus erythematosus (SLE), 10 multiple sclerosis, 2 neuromyelitis optica patients, and 17 healthy controls. The filamentous labeling pattern was observed only in brain sections treated with BS serum, and not with healthy or disease controls' serum. This was also true for scrotal skin and retinal sections treated with BS serum. This filamentous pattern, which was identified as NF-M, was formed in all BS samples, not only those with nervous system involvement. The authors suggested that NF-M may be involved in BS pathogenesis through molecular mimicry with bacterial HSP65.[62]

Gastrointestinal Involvement

The gastrointestinal involvement of BS is clinically, endoscopically, and histologically quite similar to Crohn's disease. Imamura et al.[63] studied the role of Th1 cells and HSP60 in the pathogenesis of gastrointestinal BS. They tried to see whether T-cell immune responses are skewed toward Th1 in intestinal lesions of BS patients and if this is true, to determine ectopic HSP expression and the chemokines that are involved in the Th1 cell dominance in intestinal lesions. Peripheral blood lymphocytes of 10 BS patients who were not using immunosuppressives and 10 healthy controls, as well as intestinal tissue samples of 4 BS patients, 4 Crohn's disease patients and 1 ulcerative colitis patient were studied. They observed by immunohistochemistry that the main infiltrating cells of intestinal specimens were CD4+ T-cells. In peripheral blood lymphocytes, IFN-gamma production was higher in BS patients compared to controls, and HSP60 was expressed only in BS patients and not in healthy controls. Moreover, the Th1 specific chemokine receptors CXCR3 and CCR5 were expressed more in BS patients. In the intestinal lesions also a Th1 skewed response was observed with IFN-gamma, TNF-alpha and IL12 mRNA expression and CCR5 dominance. The findings were similar to those of controls with Crohn's disease, but not the ulcerative colitis samples, which expressed CCR4 instead. Moreover, it was shown that HSP60 mRNA was expressed in intestinal samples from BS and Crohn's disease patients, but not the ulcerative colitis patient. It was suggested that HSP60 may be a trigger and a target for Th1 dominant immune responses that induce inflammation in the intestinal tract of BS patients.

Later, the same group hypothesized that TLR expressing cells could be involved in the pathogenesis of intestinal involvement of BS.[64] They studied the TLR mRNA expression in peripheral blood lymphocyte and intestinal lesion biopsies of BS patients with gastrointestinal involvement and Crohn's disease patients. They observed that TLR-2 and TLR-4 expression in peripheral blood mononuclear cells were similar in BS patients and healthy controls. In the intestinal biopsies, there was consistent expression of TLR-2 and TLR-4 in the samples from the ulcer site, whereas no expression was detected in the unaffected intestinal samples. Human HSP60 mRNA and protein which is a possible ligand of TLR-2 were again expressed in peripheral blood lymphocytes and intestinal tissues of BS patients, especially in cells accumulating in ulcerative lesions.

In a more recent study, serum HSPA6 (Heat Shock Protein Family A Member 6), which is a member of the HSP70 family, was increased in 33 intestinal BS patients compared to 40 Crohn's disease patients and 30 healthy controls. There was no correlation between HSPA6 expression levels and oral ulcers, genital ulcers, or disease activity among Crohn's disease patients.[65] Additionally, Lee et al.[66] found increased HSP27 and decreased HSP60 protein expression levels in 17 BS patients compared to 17 healthy controls, using two-dimensional (2D) electrophoresis and MALDI-TOF/TOF MS methods.

Vascular Involvement

The role of HSP in the vascular involvement of BS is less well studied.[67] Shaker et al.[67] investigated the role of HSP60, vascular endothelial growth factor and antiphospholipid antibodies in 30 BS patients and 15 healthy controls. Half of the patients with BS had vascular involvement and overall, 17 BS patients had active disease. It was observed that serum HSP60, vascular endothelial growth factor and antiphospholipid antibody levels were higher among BS patients compared to controls ($p < 0.0001$, $p < 0.001$ and $p < 0.01$ respectively). However, none of these were correlated

with disease activity. Vascular endothelial growth factor was significantly higher in BS patients with vascular involvement and those with ocular involvement. Antiphospholipid antibody levels were significantly higher in BS patients with thrombosis. Moreover, vascular endothelial growth factor and antiphospholipid antibody levels were correlated with each other. On the other hand, the serum HSP60 levels did not correlate with any of the clinical manifestations or serum levels of vascular endothelial growth factor or antiphospholipid antibodies.

Oral Bacteria

It was previously shown that several oral bacteria produce superantigens that stimulate T-cell proliferation through interaction with class II major histocompatibility complex products[14] (23). Based on earlier studies suggesting that certain antigens including superantigen and HSP may trigger cross-reactive immunopathologic responses in BS patients, Miura et al.[68] investigated the bacterial composition of subgingival plaque and saliva of BS patients and determined the level of superantigen and HSP that are produced by these bacteria. Cultures of the subgingival dental plaque samples and saliva samples of 8 BS patients yielded several bacterial species. The predominant bacteria were gram (-) short rods in subgingival plaques and *S. mitis* and *S. salivarius* in the saliva samples.

They assayed HSP production by Western blot using a polyclonal rabbit antibody to *E. coli* DnaK and a monoclonal antibody to Helicobacter pylori Gro-EL. Among the 34 strains that were studied, 27 (79%) produced HSP that reacted with the antibody against *E. coli* DnaK and 9 (27%) produced HSP that reacted with *Helicobacter pylori* Gro-EL. No superantigen activity was observed in the supernatants of the strains that were studied.

Although this study showed that bacteria isolated from the oral cavity produce HSPs and the authors suggested that this was related to BS, this study did not include any controls. It is difficult to draw conclusions about the association between HSP and oral ulcers based on these results.

Peripheral Blood Mononuclear Cells

The hypothesis of "molecular mimicry" was tested by stimulating peripheral blood mononuclear cells of 6 BS patients and 7 healthy controls with PPD or recombinant mycobacterial HSP and testing the T-cell lines that are generated for epitope specificity to human HSP peptides.[69] It was observed that the long-term T-cell lines (mainly TCR, αβCD4+ or CD8+) were highly reactive to human HSP60 derived peptides in both BS patients and healthy controls. It was suggested that these self-reactive T-cells escape central tolerance and are present in the peripheral repertoire.[70] The PPD responses in BS patients were mostly against peptide 425-41 of HSP60, which was not one of the peptides identified by the previous T-cell epitope mapping by Pervin et al.,[34] whereas response to the peptide 336-351, which seemed to be the most specific peptide for BS, turned out to be more prominent in controls. Mycobacterial HSP60 stimulation of T-cells resulted in similar levels of response against peptides 136-150, 179-197, 244-258 and 336-351. It was concluded that anti-HSP65 specific cell lines, which could also proliferate with human homologous HSP60 peptides, could be generated in both BS patients and healthy controls. However, the functional significance of these T-cell lines is not known.

A study by Kibaroglu et al.[71] failed to support the role of human HSP60 peptides as cross-reactive antigens for T-cell activation in BS. The aim was to study the effects of microbial (E. coli and S. sanguinis) and autoimmune stimulation of peripheral blood lymphocyte subsets with HSP60 in order to test whether the anti-microbial responses to streptococci were specific and whether it was associated with human HSP60 responses. They observed that after unstimulated incubation for 5 days, CD4+γδ+ T-cells, CD8+γδ+ T-cells, CD8+αβ+, CD4+CD56+ and CD8+ CD11b+ T-cell subsets were upregulated significantly more in BS patients compared to healthy controls ($p = 0.04$, $p = 0.002$, $p = 0.03$, $p = 0.03$ and $p = 0.008$ respectively). On the other hand, CD4+αβ+ T-cells were higher in healthy controls.

About microbial stimulations, CD16+ CD56+ NK cells increased significantly after *E. coli* stimulation in BS patients. CD4+CD56+ T-cells and CD8+CD11b+ cytotoxic T-cells increased after both *E. coli* and *S. sanguinis* stimulation, as well as CD8+γδ+ T-cells with *S. sanguinis* in healthy controls. The authors pointed out the fact that the subsets which are increased after stimulation with E. coli and *S. sanguinis* had already been elevated in the peripheral blood of BS patients without stimulation. This could have been associated with an in vivo activation of BS lymphocytes.

In contrast to earlier reports[72-74] they did not observe increased γδ+ T-cell subsets after *S. sanguinis* stimulation. Moreover, in contrast to the previously mentioned study by Hasan et al.[38] showing significant γδ+ T-cell responses to HSP60 derived peptides only TCR- αβ+ responses were observed against HSP60 peptides in this study. It was proposed that the elevated unstimulated γδ+ T-cell levels in BS patients could have been caused by antigens other than HSPs. The authors concluded that the responses to both E. coli and S. sanguinis extracts do not correlate with anti-HSP60 peptide responses, which exhibited themselves mainly as the presence of αβ+ T-cells.

Finally, Birtas-Atesoglu et al.[75] investigated free HSP70 and anti-HSP70 antibody levels in the sera of BS patients and compared them to rheumatoid arthritis and recurrent oral ulcer patients and healthy controls.

Free serum HSP70 levels were significantly higher among BS patients compared to healthy controls (1.82 ± 0.86 ng/ml vs. 0.67 ± 0.46 ng/ml, $p < 0.001$), but similar to the levels in recurrent oral ulcer (0.95 ± 1.01 ng/ml) and rheumatoid arthritis patients (1.1 ± 23.3 ng/ml). Serum anti-HSP levels were also significantly higher in BS patients (668 ± 658 μg/ml) compared to healthy controls (490 ± 742.1 μg/ml, $p < 0.05$) and rheumatoid arthritis patients (431.8 ± 840.9 μg/ml, $p < 0.01$) and similar to recurrent oral ulcer patients (634.7 ± 548.2 μg/ml). The serum HSP70 or anti-HSP70 levels were not correlated with demographic factors, disease duration, disease activity or treatment modalities. Moreover, no correlation was observed between anti-HSP70 and HSP70 levels in BS patients.

The significant homology between microbial and human HSPs has led to the hypothesis that bacterial HSP responsive T-cells may stimulate auto-reactive T-cells by cross-reactivity and recurrent exposure to HSPs may induce an autoimmune response to endogenous HSP.[70] The HSP responsive T-cells may produce Th1 like proinflammatory and/or inflammatory cytokines and cause tissue injury. This molecular mimicry is suggested to play a role in the development and/or activation of BS in genetically susceptible individuals.

Despite observations showing increased T and B cell responses against human HSPs and increased pro-inflammatory cytokine release, in contrast to other diseases such as juvenile idiopathic arthritis and rheumatoid arthritis where suppressive cytokines such as IL4 and TGF-β are released in response to human HSPs[54], the current data on HSPs in BS is not sufficient to conclude that HSPs play a role in the development or activation of BS.

BS has a recurrent course, where most of its manifestations disappear after a while, even if they are not treated. Relapses are common. The duration of each manifestation may vary considerably between patients and even within the same patient. The timing of tissue specimen biopsy, which may significantly affect the results, and whether patients were on any medications at that time were not mentioned in the majority of the studies. The results of peripheral blood studies may also change depending on whether the patient has active lesions or not during blood collection. Another problem is that many of the studies involving HSPs were conducted with small numbers of patients, sometimes without appropriate control groups. Moreover, whether the HSPs that are found in BS lesions have a causative role, or whether their presence is a result of ongoing inflammation, and whether they have a protective role against inflammation, as suggested in some other diseases, needs to be further studied.

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