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Behçet Disease serum is immunoreactive to neurofilament medium which share common epitopes to bacterial HSP-65, a putative trigger

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ABSTRACT

Autoimmune and dysimmune inflammatory mechanisms on a genetically susceptible background are implicated in the etiology of Behçet's Disease (BD). Heat-shock protein-65 (HSP-65) derived from Streptococcus sanguinis was proposed as a triggering factor based on its homology with human HSP-60. However, none of the autoantigens identified so far in sera from BD share common epitopes with bacterial HSP-65 or has a high prevalence. Here, we report that sera from BD patients are immunoreactive against filamentous neuronal processes in the mouse brain, retina and scrotal skin in great majority of patients. By using matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) and peptide mass fingerprinting, Western blotting and peptide blocking experiments, we have identified neurofilament medium (NF-M) as the probable antigen for the serologic response observed. Clustal Omega analyses detected significant structural homology between the human NF-M and bacterial HSP-65 corresponding to amino acids 111–126, 213–232 and 304–363 of mycobacterial HSP-65, which were previously identified to induce proliferation of lymphocytes obtained from BD patients. We also found that sera immunoreactive against NF-M cross-reacted with bacterial HSP-65. These findings suggest that NF-M may be involved in autoimmunity in BD due to its molecular mimicry with bacterial HSP-65.

1. Introduction

Behçet's disease (BD) is a systemic inflammatory disorder characterized by mucocutaneous, ocular, joint, vessel, gastrointestinal and central nervous system involvement [1]. Although its etiology is unclear, both innate and adaptive immune mechanisms triggered by environmental factors on a genetically susceptible background contribute the pathogenesis of BD. Recently, activation of the inflammasome and downstream inflammatory pathways have emerged to play important roles in BD [2]. In parallel with several lines of evidence suggesting defects in sensing and degrading pathogens as well as in regulation of innate and adaptive immune responses, recent genome wide association studies (GWAS) from Turkish, Japanese, Chinese and Korean patients disclosed single nucleotide polymorphisms at Familial Mediterranean Fever (MEFV), Nucleotide-Binding Oligomerization Domain Protein 2 (NOD2), Toll-Like Receptor 4 (TLR4), genes involved in inflammation and pathogen degradation [3].

Triggers of bacterial (mainly *Streptococcus sanguinis*) [4] or viral (mainly Herpes Simplex Virus 1) [5] origin have long been considered in the pathogenesis of BD. Heat-shock protein-65 (HSP-65) derived from oral *Streptococcus sanguinis* was proposed to play important roles as a triggering extrinsic factor in BD based on its high homology with human 60-kDa HSP (HSP-60) [6]. Cross

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reactivity between human and bacterial HSP may induce proliferation of autoreactive T cells and initiate (or contribute to) the immune dysregulation seen BD [7]. However, despite the fact that several autoantigens were identified in sera of small groups of BD patients over the last decades, none of them has been reported to have a high prevalence or share a common epitope with bacterial HSP-65. Hence, we planned to search for a novel autoantigen in BD serum by incubating mouse tissue sections with patient sera, a method that has been instrumental to disclose several autoantigens with help of indirect fluorescence microscopy.

Here, we report that sera from BD patients are immunoreactive against filamentous neuronal processes in the mouse brain, retina and scrotal skin in majority of BD patients. By using matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) and peptide mass fingerprinting (PMF), Western blotting and peptide blocking experiments, we have identified neurofilament medium (NF-M) as the probable antigen for the serologic response observed in BD patients. Clustal Omega analyses detected significant structural homology between the human NF-M (P07197) and bacterial HSP-65 corresponding to amino acids 111–126, 213–232 and 304–363 of mycobacterial HSP-65 (P9WPE9), which (and their human homologues) were previously identified to induce proliferation of lymphocytes obtained from BD patients [6–10]. We also found that sera immunoreactive against the NF-M cross-reacted with bacterial HSP-65.

2. Materials and methods

2.1. Patients and samples

A total of 75 sera were studied. Sera from 34 BD patients (23 male, 11 female and; 14 with neurological involvement), 12 systemic lupus erythematosus (SLE; none of the SLE patients had neuropsychiatric manifestations, 11 female, 1 male), 10 Multiple Sclerosis (MS) (5 male, 5 female), 2 Neuromyelitis optica (NMO, 2 female) patients and 17 healthy controls (10 female, 7 male) were recruited from neurology and rheumatology clinics of Hacettepe University Hospitals. The demographic and clinical features of BD patients are summarized in Table 1. The study was approved by the Ethics Committee of Faculty of Medicine, Hacettepe University, Ankara, Turkey (GO 14/361-05, FON 06/40-25).

All of the BD patients fulfilled the previously described diagnostic criteria. None of the participants had a history of an autoimmune disease other than BD. SLE patients were classified according to American College of Rheumatology revised criteria. MS patients were all relapsing-remitting type, on IF-β1a and IF-β1b treatment and blood samples were collected in steroid-free clinical remission period. NMO patients had a typical clinical presentation with optic neuritis as well as longitudinally extensive transverse myelitis on MRI. Their sera tested positive for anti-AQP4 antibody and exhibited the characteristic perivascular immunostaining of mouse brain sections.

Sera obtained from subjects were kept frozen at −80 °C until assayed. Freshly isolated brain, scrotal skin and eye sections (20 μm) from Swiss albino mice as well as primary mouse cortical neuron cultures were used for indirect immunofluorescence labeling. Tissue sections were kept at −20 °C until immunolabeling with sera. Cultured neurons were preserved at +4 °C in PBS solution after 4% paraformaldehyde fixation until immunolabeling performed within 2 days. Immunolabeling with sera was performed at room temperature (+23 to +25 °C).

2.2. Pre-absorption

Since serum samples contain many non-specific antibodies, preabsorption of patient and control sera with lyophilized guinea pig liver at 1/60 dilution in phosphate buffered saline (PBS) solution was performed as described by Lennon and co-workers ([11]) to minimize non-specific background staining. Pre-absorption was applied to all samples before immunostaining experiments.

2.3. Incubation of tissue sections with sera

For indirect immunofluorescence assay, 20 μm-thick mouse tissue sections (brain, eye, scrotal skin) or human hippocampal brain sections resected during epilepsy surgery (obtained from the tissue bank of Hacettepe University Biobank, a Eurobiobank member) were cut on a freezing cryostat (Leica), fixed with 10% formaldehyde in PBS solution for 4 min and permeabilized with 1% CHAPS solution (AppliChem) in PBS. Sections were blocked with 10% normal goat serum (Millipore) at room temperature for 60 min and then incubated with pre-absorbed patient and control sera at 1/60 dilution (in PBS) for 70 min at room temperature (in initial experiments for 60 min). Secondary labeling was done with fluorescent-conjugated (FITC) goat anti-human IgG antibody (1/200 dilution, Jackson Immunoresearch) at room temperature for 60 min (in initial experiments at 1:500 dilution for 70 min). Slides were washed 3 times for 5 min with PBS between the each step above in order to minimize non-specific background binding. After immunofluorescent labeling, brain sections were mounted with Hoechst solution (Hoechst 33258, Molecular Probes, 10 mg/ml in PBS) on microscope glass slides. For direct immunofluorescence assay of NF-M, mouse tissues blocked with %10 normal mouse serum in PBS-T for 60 min at room temperature and, an anti-NF-M antibody (mouse monoclonal, Abcam ab7794) was used at 1/500 dilution in the same blocking solution at +4 °C overnight. Secondary antibody labeling was done with Cy3 goat anti-mouse (1/200 dilution in blocking solution, Jackson Immunoresearch) at room temperature for 90 min. For double immunolabeling with lectin, sections were incubated in Texas Red Lycopersicon esculentum (Tomato) Lectin (1/1000 in PBS, Vector Laboratories, TL-1176) at 4 °C overnight and then cover-slipped with Hoechst after 3 washes with PBS. Slides were evaluated under wide angle fluorescent (Nikon Eclipse E600) and laser scanning confocal microscopes (Zeiss LSM- 510, Leica SP8).

2.4. Incubation of neuron cultures with sera

For primary neuron cultures, cerebral cortical neurons were harvested from brains of 17/18-day-old mouse embryos. The meninges were carefully removed and cerebral cortices were isolated. Harvested brain tissue was minced, washed 2–3 times with cold PBS and, treated with 1:1 trypsin (2.5%): DMEM/F-12 medium at 37 °C for 20–30 min. Trypsin was removed via centrifugation at 1200 RPM (4 °C) for 3 min and after the aspiration of the supernatant, the pellet was washed 2–3 times with DMEM/F-12 medium in 10% FBS/PS. 125 μg of DNase in DMEM/F12 with 10% FBS solution was applied at 37 °C for 5 min and then the tissue was homogenized by pipetting. The homogenized brain tissue was centrifuged at 1400 RPM (4 °C) for 3 min. Cortical cells were isolated and plated on poly-D-lysine coated cover slips at 1×10^5 cells/cm^2 concentration. The culture medium was composed of Neurobasal Medium (Invitrogen) containing B27 supplement (Invitrogen), penicillin (50 U/mL), streptomycin (50 μg/mL), 2 mM L-Glutamine, 10 μM β-mercaptoethanol and 25 μM glutamate. Two days later, half of the medium was changed with DMEM comprising 10% fetal bovine serum, 1% P/S and 10 μM cytosine β-D-arabinofuranoside hydrochloride (AraC, Sigma C6645) for elimination of glial cells. The culture medium was changed back to Neurobasal Medium containing B27, P/S, 2 mM L-Glutamine 24 h later. Then, half of the

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