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Detection of heat shock proteins but not superantigen by isolated oral bacteria from patients with Behcet's disease

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We isolated bacteria from periodontal sites and mixed saliva in eight patients with Behcet's disease and surveyed them to determine whether they produced heat shock proteins (HSPs) and superantigen. Cultivable bacterial compositions from periodontal sites and saliva were examined by anaerobic culture using blood agar plates. Gram-negative anaerobic rods such as *Prevotella intermedia, Fusobacterium nucleatum*, and *Capnocytophaga* species were predominant in the isolates from the subgingival plaque samples. The *Streptococcus mitis* group was the most common type isolated from the saliva samples. To detect the production of HSPs, Western blot analyses were performed using a polyclonal rabbit antibody to *Escherichia coli* DnaK and a monoclonal antibody to *Helicobacter pylori* Gro-EL. Sonic extracts of 27 of the strains (79.4%) reacted with the antibody against *E. coli* DnaK. Nine of these 34 strains (26.5%) were found to produce HSPs that reacted with antibody to *H. pylori* Gro-EL. A total of 54 isolates were examined for superantigen activity against human peripheral leukocytes. Twenty-five gram-negative clinical strains isolated from chronic periodontitis lesions and 20 ATCC strains of microorganisms were also examined. We could not detect any superantigen activity in 500× diluted supernatant of the strains isolated from the eight patients with Behcet's disease. The present study indicates that the anaerobic strains isolated from the oral cavity of these patients produce HSPs, the production being related to Bechet's disease.

Key words: Behcet's disease; heat shock proteins; oral anaerobe; superantigen

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Behcet's disease is an inflammatory disorder of unknown etiology characterized by recurrent oral aphthous ulcers, genital ulcers, uveitis, and skin lesions (14, 27). Microbial infections have been implicated in its development (14, 18). Herpes simplex virus DNA and serum antibodies against the virus have been found in a higher proportion of patients with Behcet's disease than in controls (14). The *Streptococcus mitis* group has been suggested as a causative agent, because these bacteria and the antibodies against them are frequently found in the oral flora and serum, respectively, of patients with the disease (16, 22, 23). However, none of these infectious agents has been proven to cause Behcet's disease.

It is well known that microbial infections can moderate host responses. The production of superantigens and heat shock proteins (HSPs) by infected microorganisms is closely associated with such changing host responses (3, 6, 34, 35, 37, 38). A series of studies has led to the hypothesis that specific antigens including superantigen and HSPs of microorganisms may trigger cross-reactive immunopathologic responses in patients with Behcet's disease (5, 9, 10, 15, 26, 31, 32). In fact, several oral bacterial species including the *S. mitis* group (16, 22, 23), *Prevotella intermedia* (20), *Porphyromonas gingivalis* and *Actinobacillus actinomycetemcomitans* (7) have been shown to produce superantigens that stimulate T cells to

proliferate nonspecifically through interaction with class II major histocompatibility complex products. HSPs are highly conserved immunogenic proteins that are often immunodominant antigens produced in bacteria and mammalian cells by a variety of stresses (37, 38). Many research groups have indicated that HSPs have been implicated in the etiology of Behcet's disease (5, 9, 15, 19, 26).

In the present study, we investigated the bacterial composition of the subgingival plaque and saliva collected from patients with Behcet's disease and determined the levels of superantigen and HSP production by the oral bacteria isolated from these patients.

Materials and methods

Subjects

After their informed consent was obtained, eight patients (five men and three women) with Behcet's disease who consulted the Second Hospital of Tokyo Women's Medical University, School of Medicine for periodontal treatment were enrolled in the present study. The mean patient age was 44.4 years (32–61 years). These patients had not received any antibiotics within the previous 4 weeks.

Sampling of dental plaque and saliva

Sites for sampling subgingival dental plaque were isolated with sterilized cotton rolls. Subgingival plaque samples were collected with a sterilized scaler. Mixed saliva samples were also obtained. All samples were immediately transferred into 0.9 ml RTF transport medium (29), diluted in RTF from 10^{-1} to 10^{-7}. Then 100 μl aliquots of each dilution were incubated on Tryptic soy agar plates (BBL Microbiology Systems, Cockeysville, MD) with 10% horse blood, hemin (5.0 μg/ml), and menadione (0.5 μg/ml) and incubated in an anaerobic chamber containing 10% CO_2, 10% H_2, and 80% N_2 for 7 days.

Identification

Colonies from an appropriate number of plates were picked, purified by repeated transfer, and characterized. Colony morphology, gram-staining, cell morphology, motility, and aerobic growth were checked. Biochemical tests included indole production, esculin hydrolysis, nitrate reduction, gelatinase activity, catalase, and fermentation of glucose, lactose, sucrose, cellobiose, and mannitol and the BAPNA test for trypsin-like enzyme described by Loesche (21). The enzymatic activity of the examined strains was also evaluated by the API ZYM system (Bio Merieux S.A. Marcy-L'Etoile, France).

Assay for superantigen production

The cultured supernatants of the isolated strains were examined for the production of superantigen. Twenty ATCC strains of *Actinomyces naeslundii* 15987 and 12104, *Actinomyces israelii* 12102, *Propionibacterium acnes* 11827 and 11828, *A. actinomycetemcomitans* 43718, 33384 and 29523, *P. intermedia* 25611, *Prevotella nigrescens* 33563, *P. gingivalis* 33277 and 53977, *Fusobacterium nucleatum* 25586, *Campylobacter rectus* 33238, *Tannerella forsythia* 43037, *Eikenella corrodens* 23834, and *Treponema denticola* 33521 and 35405, *Mycoplasma penetrans* 15845, and *Mycoplasma buccae* 14851 were also included in this experiment. The Tryptic soy broth culture media for gram-positive and gram-negative bacteria, the TYGVS broth for *T. denticola* strains described in our previous paper (12) and *Mycoplasma* broth (BBL Microbiology Systems) for *Mycoplasma* species were used. Culture supernatants were filtered through 0.1 μm sterile Millex-VV filters (Millipore, Bedford, MA) and evaluated for superantigen activity. The assay for superantigen production was carried out as described by Uchiyama et al. (35). Staphylococcus enterotoxin A (SEA; Toxin Technology, Inc., Miami, FL) was used to stimulate lymphocyte culture as a positive control at a concentration of 400 ng/ml. For assay purposes, the appropriate concentration of SEA as a positive control or broth alone as a negative control was added to the cells. Heparinized venous blood (30–50 ml) from healthy volunteer donors was diluted with phosphate-buffered saline (PBS) and layered over Ficoll–Conray medium containing 100 g of Ficoll 400 medium (Amersham Biosciences, Piscataway, NJ) and 240 ml of Conray 400 medium (66.8% w/v, Daiichi Seiyaku, Tokyo, Japan) per liter, a mixture whose density had previously been adjusted to 1.077 μg/ml with saline. The tubes were centrifuged for 30 min at 500 × g at room temperature. Leukocytes were harvested from the Ficoll–Conray interface and washed twice to remove the Ficoll medium with Hanks' solution (Nissui, Tokyo, Japan) containing 2% fetal calf serum. Cells were collected by centrifugation at 200 × g for 10 min at 4°C, and then resuspended in the RPMI 1640 culture medium with the supplementation of 100 μg/ml streptomycin and 100 IU/ml penicillin. For the superantigen assay, cells were distributed at 1×10^5 viable cells per well in 96-well round-bottomed microplates (Iwaki, Chiba, Japan). After incubation at 37°C in a humidified environment containing 5% CO_2 for 2 days, the cultures were pulsed with methyl-^{3}H thymidine (1 μCi/well) for the last 16 h of incubation, and thymidine incorporation was determined in a liquid scintillation counter. The data are presented as averages of triplicate cultures.

Heat shock protein production of isolates from Behcet's patients

To detect the production of HSPs, Western blot analyses were performed using polyclonal rabbit antibody to *Escherichia coli* DnaK (Hsp 70, Upstate Biotechnology Inc., Waltham, MA) and a monoclonal antibody to *Helicobacter pylori* Gro-EL (Hsp 60, Wako Pure Chemical Industries, Ltd, Osaka, Japan). Cells grown in each medium were harvested by centrifugation and washed twice with PBS (pH 7.2). The cell suspension was homogenized with a sonicator (Branson, Danbury, CT) at 100 W for 5 min on ice, and the supernatants were used in the experiment. These sonic extracts of bacterial strains were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) according to the method described by Towbin et al. (33). Separated proteins by SDS-PAGE were transferred to PVDF membranes, and the membranes were then washed twice and incubated with rabbit anti *E. coli* DnaK antibody or mouse anti-*H. pylori* Gro-EL antibody. After washing, the membranes were reacted with peroxidase conjugated goat anti-rabbit IgG antibody or goat anti-mouse IgG antibody. The peroxidase reaction was initiated with Tris buffered saline containing 4 methoxy-1-naphthol and 0.02% H_2O_2.

Results

Bacterial flora of dental plaque and saliva

The bacterial composition of samples obtained from eight patients with Behcet's disease were examined by culture on blood agar plates in an anaerobic chamber. The cultivable compositions of the subgingival plaque and mixed saliva are summarized in Tables 1 and 2, respectively.

The predominant strains from the subgingival plaque samples were groups of *S. mitis* and *Streptococcus salivarius*. Isolated strains of black-pigmented colonies on blood agar in subgingival plaque

Table 1. Comparison (%) of anaerobically cultivable bacteria in samples of subgingival dental plaque obtained from patients with Behcet's disease

	Patients							
Nos.	1	2	3	4	5	6	7	8
Age	37	42	40	51	41	51	32	61
Sex	F	M	M	M	F	M	M	M
Bacterial species in dental plaque	Detection rate (%)							
Streptococcus mitis group	20.2	21.4	9.1	20.1	1.9	–	26.1	17.5
Streptococcus salivarius group	19.0	8.6	12.0	–	–	–	4.3	10.0
Streptococcus mutans group	–	–	–	–	–	–	4.3	–
Actinomyces naeslundii	3.7	10.0	6.0	6.7	–	23.0	13.0	5.0
Actinomyces israelii	–	–	–	–	4.5	–	–	–
Porphyromonas gingivalis	–	–	4.0	20.8	–	–	–	–
Prevotella intermedia	5.1	4.3	6.8	5.4	1.0	–	–	–
Prevotella melaninogenica	–	–	0.9	10.7	22.2	–	8.7	7.5
Prevotella denticola	–	–	–	–	–	3.8	–	–
Prevotella species	–	7.1	6.4	–	–	11.5	–	12.5
Fusobacterium species	8.9	–	1.6	–	–	15.4	–	–
Fusobacterium nucleatum	–	2.8	2.0	7.3	–	7.7	4.3	–
Capnocytophaga species	7.6	7.1	1.5	–	11.2	–	4.3	5.0
Leptotrichia buccalis	–	2.9	2.9	1.0	2.9	7.7	–	–
Eikenella corrodens	–	2.9	–	2.6	–	–	–	10.0
Selenomonas sputigena	–	–	5.0	–	–	–	4.3	–
Veillonella species	1.3	2.9	4.0	7.9	4.0	–	–	–
Unidentified	34.2	30.0	37.8	17.5	52.3	30.9	30.7	32.5

Table 2. Comparison (%) of anaerobically cultivable bacteria in samples of mixed saliva obtained from patients with Behcet's disease

		Patients							
	Nos.	1	2	3	4	5	6	7	8
	Age	37	42	40	51	41	51	32	61
	Sex	F	M	M	M	F	M	M	M
Bacterial species in dental plaque	Detection rate (%)								
Streptococcus mitis group		30.3	25.0	4.7	14.3	10.6	–	77.5	70.0
Streptococcus salivarius group		22.2	21.7	68.1	7.1	9.1	8.8	–	10.0
Veillonella species	–	–	–	7.1	–	–	–	–	
Actinomyces naeslundii		–	–	–	14.3	18.2	–	–	–
Fusobacterium nucleatum		3.0	–	–	–	–	–	–	10.0
Capnocytophaga species		2.0	–	–	7.1	–	–	–	–
Prevotella intermedia	–	–	–	–	–	2.9	–	–	
Prevotella species	–	–	4.7	–	–	23.5	3.8	–	
Selenomonas sputigena		–	–	–	–	–	–	3.8	–
Unidentified	42.5	53.3	22.5	50.1	62.1	64.8	14.9	10.0	

samples from Behcet's patients were identified as *P. intermedia*, *Prevotella melaninogenica* and *P. gingivalis*. However, many of the isolated strains of gram-negative short rods could not be identified by the biochemical characteristics surveyed in this study. More than 10% of the total colony forming units (CFUs) in subgingival samples from patient Nos. 2, 3, 4, 5, 6, and 8 were *Prevotella* species. In addition, we found more than 20% of *P. gingivalis* in the samples obtained from patient No. 5. Genus *Fusobacterium*, including *F. nucleatum* and *Capnocytophaga* species, were also isolated from almost every patient at a moderate detection rate. Most of the isolates in the saliva samples were *Streptococcus* species, mainly groups of *S. mitis* and *S. salivarius*. We could not identify all of the strains isolated from each sample.

Superantigen production

We examined 54 strains isolated from the oral cavities of eight patients with Behcet's disease for their ability to produce superantigen using healthy volunteers' lymphocytes. These 54 strains included 8 strains of the *S. mitis* group, 6 strains of the *S. salivarius* group and 3 strains of the *Streptococcus mutans* group, 4 strains of unidentified *Streptococcus* species, 2 strains of *Veillonella* species, 6 strains of *A. naeslundii*, one strain of *A. israelii*, 2 strains of *P. gingivalis*, 4 strains of *P. intermedia*, 2 strains of *Prevotella denticola* and an unidentified *Prevotella* species, 6 strains of *F. nucleatum*, 4 strains of unidentified *Capnocytophaga* species, 2 strains of *Leptotrichia buccalis*, and one strain each of *E. corrodens* and *Selenomonas sputigena*. The superantigen production was examined using 50× and 500× dilution of the culture supernatant from each culture. The 50× diluted culture supernatant sample from some strains of *Streptococcus* species induced weak thymidine incorporation. However, repeated examinations using 500× diluted culture supernatant sample failed to find superantigen activity in strains isolated from the oral cavities of the eight Behcet's patients.

We also examined the production of superantigen by 25 gram-negative clinical strains isolated from chronic periodontitis lesions and 20 ATCC strains. No superantigen production was found in strains of *A. actinomycetemcomitans*, *P. gingivalis*, *P. intermedia, P. nigrescens, C. rectus, E. corrodens, F. nucleatum, P. intermedia, T. denticola, T. forsythia, M. buccae* and *M. penetrans*.

Production of HSPs by isolated bacterial strains

The HSPs produced that reacted with the polyclonal antibody against *E. coli* DnaK (HSP 70) and with the monoclonal antibody against *H. pylori* Gro-EL (HSP 60) are summarized in Table 3. The strains examined were 4 strains of *P. intermedia*, 3 strains of *F. nucleatum*, 2 strains each of *S. sputigena* and *L. buccalis, and* one strain each of *P. denticola, P. gingivalis*, and *E. corrodens.* Ten unidentified strains of *Prevotella* species, 5 strains of unidentified *Streptococcus* species, 3 of *Capnocytophaga* species, 1 of *Veillonella* species, and 1 unidentified gram-negative rod were also examined. Twenty-seven strains (79.4%) reacting with anti-*E. coli* DnaK and 9 strains (26.5%) reacting with anti-*H. pylori* Gro-EL were found among 34 strains in the present study. We confirmed these results with repeated Western blot analysis.

Discussion

Oral ulceration is usually an initial symptom and is seen in all patients at some time in the reported clinical course of Behcet's disease (25). To clarify the relationship between the oral ulceration and oral bacterial interactions, including the immunopathologic factors, we attempted to isolate

Table 3. Reactivity of bacterial strains isolated from samples of subgingival plaque and mixed saliva from patients with Behcet's disease against HSPs of *E. coli* DnaK and *H. pylori* Gro-EL

	Number of positive strains/Number of examined strains	
Bacterial species	anti-*E. coli* DnaK	anti-*H. pylori* Gro-EL
Prevotella intermedia	3/4	1/4
Prevotella denticola	0/1	0/1
Unidentified *Prevotella* species	10/10	4/10
Porphyromonas gingivalis	0/1	0/1
Eikenella corrodens	1/1	0/1
Fusobacterium nucleatum	2/3	0/3
Unidentified *Capnocytophaga* species	2/3	1/3
Leptotrichia buccalis	2/2	0/2
Selenomonas sputigena	1/2	2/2
Unidentified *Veillonella* species	1/1	0/1
Unidentified gram-negative rod	1/1	0/1
Unidentified *Streptococcus* species	4/5	1/5
Total	27/34	9/34

and detect superantigen-producing anaerobic bacteria in subgingival dental plaque and saliva from patients with Behcet's disease. We isolated 620 strains from eight patients. Based on gram-staining, cell morphology, biochemical properties, and the results of the anaerobic API enzymatic kit, we identified most of the isolated strains at mainly the genus level. The anaerobic bacterial compositions of the subgingival plaque and saliva are listed in Tables 1 and 2, respectively. We obtained the subgingival plaque from inflammatory gingival sites but for ethical reasons did not record the periodontal status with probes or X-ray photographs. The predominant cultivable bacteria in the subgingival plaque samples were gram-negative short rods. A comparison of the present culture study with cultivable findings of subgingival plaque reported previously by Sutter et al. (30) and Umeda et al. (36) revealed no significant differences. The predominant bacterial composition of mixed saliva in the present study comprised *Streptococcus* species. The present findings are similar to those in a previous cultivable study (24). No specific species or inherent bacterial composition was found in the present study.

Immunomodulatory effects, especially lymphocyte stimulation, are thought to be implicated in the development of Behcet's disease (9, 18, 19, 27, 28). It has been found that *S. mitis* produces superantigen, and an association of extracellular products of oral *Streptococcus* with the pathogenesis of oral mucosal diseases has also been suggested (16, 22, 23). Periodontal disease-associated bacteria such as *P. gingivalis* produce substances with some of the characteristics of superantigens (7), and certain *P. intermedia* have been suggested to activate Vβ-specific T cells in a manner similar to that of other known microbial superantigens (20). We examined the culture supernatants of 54 isolated bacterial strains from eight patients with Behcet's disease, 25 gram-negative strains from chronic periodontitis lesions and 20 ATCC oral bacterial strains in order to detect superantigen, but none of the strains we examined produced any superantigen in the culture supernatant, although 50× diluted sample from some strains of *Streptococcus* species induced weak thymidine incorporation. Further study using concentrated culture supernatant is required to detect the superantigen activity from these strains.

We have previously reported that oral *Mycoplasma* species isolated from HIV-seropositive patients did not produce superantigen (2). It is possible that some culture conditions or stages affect superantigen production. Recent studies have indicated that dental plaque microorganisms form a biofilm with a complex bacterial community that can modulate pathogenic factors (4, 17). In this study, we examined planktonic bacterial cells grown in broth culture. It is possible that subgingival plaque bacteria form biofilms that then produce immunomodulating factors such as superantigen *in vivo*. Further analysis is required to examine the superantigen production of both intact dental plaque and its isolates obtained from patients with Behcet's disease in advancing stages of the disease under various culture conditions.

HSPs have been called 'common antigens' and have been implicated in immunomodulatory actions such as immunosuppression and the induction of autoimmune diseases (37, 38). One immunohistochemical study of HSPs revealed that they are expressed differently in experimental cells of patients with systemic lupus erythematous and atopic dermatitis (8). There are two kinds of responses to HSPs: the response to the infectious pathogen and the recognition of the conserved epitope, which is thought to play some role in autoimmune disease and has therefore been called the 'common antigen'. In addition, T cells with γδTCRs, which are abundant in mucosal membranes, exhibit unusually high reactivity with some HSPs. Oral streptococcus antigens such as HSPs are involved in the etiology of Behcet's syndrome (18, 19, 26, 28). The present study showed that many oral bacterial species isolated from patients with Behcet's disease produce HSP70. We have previously reported that strains isolated from patients with chronic periodontitis with pustulosis palmaris et plantaris (PPP), and HIV-seropositivity, produce HSPs (1, 2, 11). We have also examined the relationships between the onset of PPP, periodontitis and HSP levels of IgG against HSPs, including *A. actinomycetemcomitans* DnaJ, in sera (11). In that study, we found that periodontal therapy and extraction of teeth with periapical infection resulted in remission of PPP and a statistically significant reduction in the levels of IgG against HSPs.

In the present study, the mean ratios of HSP-producing bacterial strains that reacted with both antibodies were significantly lower than those in the strains isolated from patients with Behcet's disease we examined previously. We previously found that *C. rectus* strains isolated from adult periodontitis lesions possessed cross-reacting antigens including HSP cross-reactive with *H. pylori* strains (13). As infectious disease progress, it is possible that immune responses to HSPs may be initiated, and it is also possible that the differences we note may be related to immune suppression. The results of the present study do not provide sufficient data to allow discussion of the relationship between bacterial infections in the oral cavity and the progression of Behcet's disease. However, it is still possible that HSPs produced by microorganisms in the oral cavity may lead to the acceleration of oral membrane ulceration in patients with Behcet's disease.

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