

## Behcet's Disease and Related Diseases -Immune Reactions to Oralstreptococci in Their Pathogenesis

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### ABSTRACT

Behçet's disease (BD) is a systemic disorder characterized by the recurrent involvement in the muco-cutaneous, ocular, intestinal, vascular, and/or nervous system organs. The clinical muco-cutaneous manifestations including recurrent aphthous stomatitis (RAS), erythema nodosum (EN) -like eruption, genital ulceration, etc. of patients with BD were reviewed in their pathogenesis comparing with the similar symptoms seen in patients without BD (non-BD). Most of BD patients tend to have hypersensitivity against *streptococci* which might be acquired in the oral cavity through the innate immune mechanism. Generally, BD patients have the systemic symptoms following RAS symptom as an immune reaction. Then, the characteristics of hypersensitivity to oral *streptococci* may be utilized in order to make a diagnosis for BD. The skin prick with self-saliva including oral *streptococci* was much more sensitive than "Pathergy test" conventionally used for BD diagnosis. HLA-B51-restricted CD8+ T cell response is suspected to catch the target tissues expressing major histocompatibility complex class 1 chain-related gene A (MICA) by stress in active BD patients. *Bes-1* gene and 65kD of heat shock protein (HSP-65) derived from *Streptococcus sanguinis* (*S. sanguinis*) are detectable in the lesions. The peptides of *Bes-1* gene are highly homologous with

the retinal protein Brn3b which might be connected with the eye involvement in BD patients. Also, the peptides seem to be homologous with HSP-65 in association with the human HSP-60 which reactively appeared in serum of the patients involved by *S. sanguinis*.

Then, the pathogenesis of BD was conclusively discussed on the relationship between RAS and the systemic symptoms by the vascular reaction due to immune responses against antigens derived from *S. sanguinis*. Non-BD patients with RAS and/or Lipschutz genital ulceration were weekly sensitized by oral streptococci, except for patients with EN.

**Key words:** Behçet's disease; *Bes-1* DNA; Heat shock protein (HSP); Recurrent aphthous stomatitis (RAS); Salivary prick test

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### INTRODUCTION

Behçet's disease (BD)<sup>[1]</sup> is a chronic systemic inflammatory disorder characterized by the recurrent involvement of muco-utaneous [recurrent aphthous stomatitis (RAS), genital ulceration, erythema nodosum (EN)-like eruption, acne-like eruption, etc.], ocular, vascular, digestive and/or nervous system organs. RAS showing oral aphthous ulceration generally starts as an initial sign since childhood and/or youth before the systemic symptoms of BD patients<sup>[2-4]</sup>. Although the actual etiology of BD is still unclear, the pathogenesis has been generally clearer by the etiological studies based on the genetic intrinsic factors and extrinsic triggering factors<sup>[5-16]</sup>. As one of the triggering factors, the oral unhygienic condition may be suspected, because periodontitis, decayed teeth, chronic tonsillitis, etc. are frequently noted in BD patients<sup>[12,13]</sup>. The proportion of *Streptococcus sanguinis* (*S. sanguinis*), which was previously recognized as *species* of the genus *Streptococcus* named "*S. sanguis*", was significantly high

in the oral bacterial flora of BD patients in comparison with those of healthy controls. *S. sanguinis* from BD patients was identified as uncommon serotype KTH-1 (so-called BD113-20) by the bacterial and enzymatic properties<sup>[14-16]</sup>. Most of BD patients tend to acquire hypersensitivity against *streptococci* in their oral flora, as previously demonstrated that the cutaneous reactions by the injection and/or prickle with bacteria antigens of *streptococci* and enterococci were much stronger than the reaction by "Pathergy test". The histology from the cutaneous streptococcal response of a BD patient is similar to the vascular reaction seen in EN-like eruption (Figure 1a-c)<sup>[11,12,17]</sup>. The cutaneous reactions to streptococcal antigens induced the clinical symptoms in some BD patients<sup>[18]</sup>.

Non-BD patients with RAS (non-BD RAS) were also considered to react with streptococcal antigen<sup>[19]</sup>, although several environmental factors are also to be a trigger of aphthous ulceration<sup>[20]</sup>. In vitro system, inflammatory cytokines, interleukin (IL)-6 and interferon (IFN)- $\gamma$  were produced from peripheral blood mononuclear cells (PBMCs) of BD patients by stimulation with streptococcal antigen<sup>[21]</sup>. The titers of serum-antibody against *streptococci* were also elevated in BD patients<sup>[22]</sup>. The 65kDa of heat shock protein (HSP-65) derived from *S. sanguinis*, can be detected along with counterpart human HSP-60 which reactively appears in the sera and lesions of BD patients. The peptides of HSP-65 show considerable homology with those of the human HSP-60<sup>[23-25]</sup>.

Epidemiology surveys suggest that the prevalence of BD is highly distributed from the Mediterranean countries to Japan via China and South Korea, along so-called "old Silk Route". The prevalence rates

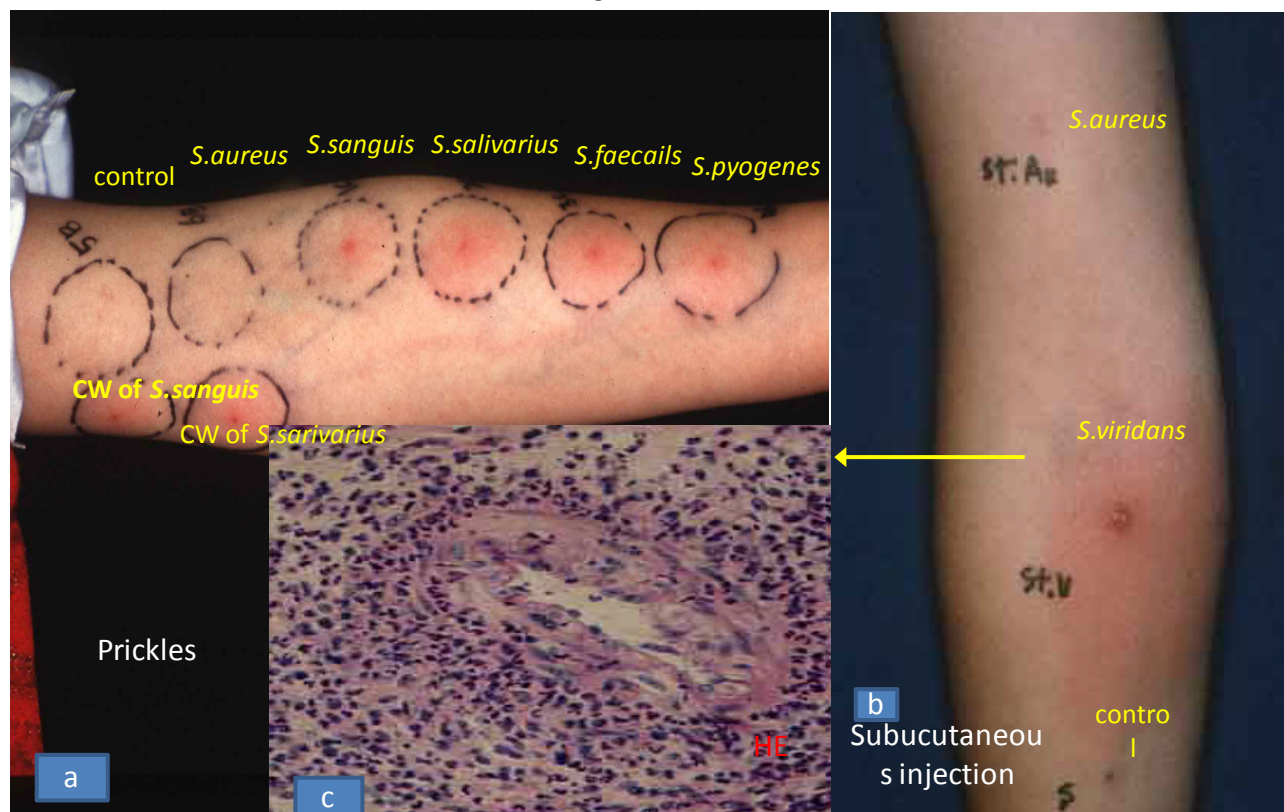
of 1990s were 8 to 37 per 100,000 in adult population of Turkey and 11 -13per 100,000 population in Ningxiahui and Heilongjiang of China<sup>[26,27]</sup>. In Japan, the prevalence was suggested to be 13 per 100,000 as well as in Korea in the 1970s<sup>[2,4]</sup>, but its rate has decreased lately, because the environmental conditions, such as oral health behaviors, etc., are changed<sup>[28]</sup>.

Then, we have attempted to review about the new diagnostic ways for BD in comparison to the related diseases showing the similar symptoms due to immunological reactions.

## MUCOCUTANEOUS INVOLVEMENTS

**RAS:** The oral aphthous ulceration punch-out shaped painfully occurs on the tongue, buccal mucosa, gingival and lip, continues around a week, though self-limited, and nearly 100% of BD patients will be associated as the initial signs<sup>[1-4]</sup> (Figure 4 and 5). On the other hand, non-BD RAS is a very common disorder due to trauma, some viral and/or bacterial infections and other autoimmune diseases, because about 20% of the general population is thought to be affected in the world<sup>[20,29,30]</sup>. The biopsy specimen from RAS lesion of a BD patient revealed the epithelial cells surrounded by neutrophils and T cells like the antibody dependent cell mediated cytotoxicity. The epithelial cells of the ulcer margin were stained with anti-human IgA, IgM, complement, streptococcal antibodies and HLA-DR monoclonal antibody<sup>[17,31]</sup>. However, it is difficult to differentiate oral ulceration-lesions in patients with BD from non BD-RAS by the clinical and/or histological aspects.

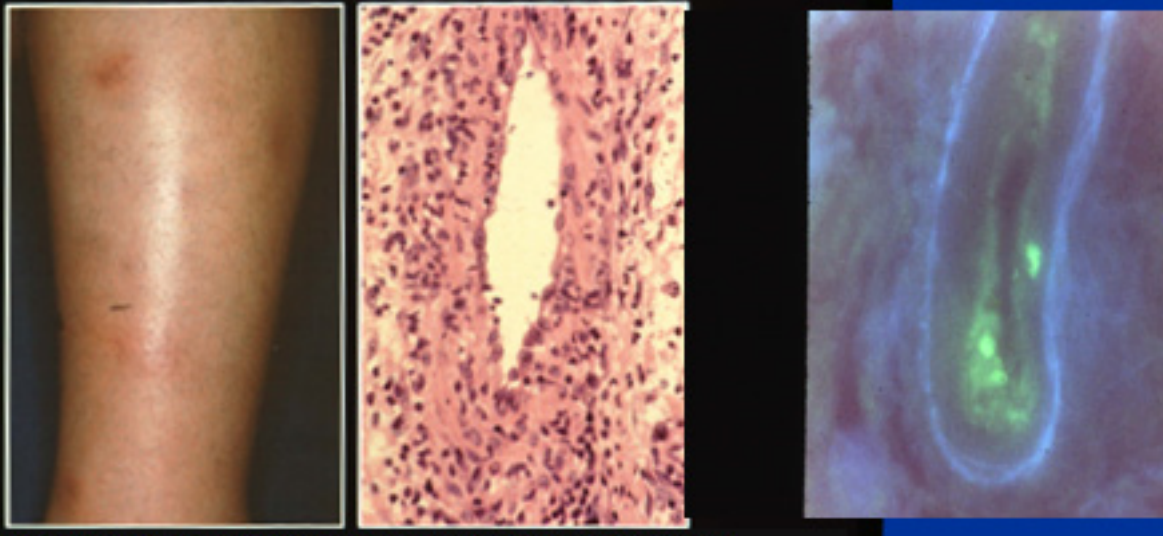
Cutaneous tests with bacterial antigens. Kaneko, et al: Yonsei Med J 38: 444, 1997



**Figure 1** a. Prick tests by bacterial antigens ( $1 \times 10^9$  org./mL) (Hollister-Stairs, USA). After 24-48 h, strong erythemaous reactions appeared by *Streptococcus* (*S.* *sanguis*, *S.sarivarius*, *S.faecalis*, *S.pyogenes* and cell wall of *S.sanguis* and *salivarius* antigens except *Staphilococcus* (*S.* *aureus*) antigen and saline (control). b. The cutaneous reaction by 0.01 mL injection of *S.viridans* and *Staphilococcus aureus* antigens after 48 hours. c. A biopsy specimen from the reactive site by *S.viridans* showed vascular phenomenon was similar to that of EN-like eruption of a BD patient.

## Histology and immunohistology by anti-streptococcal group D antibody in the lesions of Behcet's disease (Kaneko, et al: Br J Dermatol, 1985)

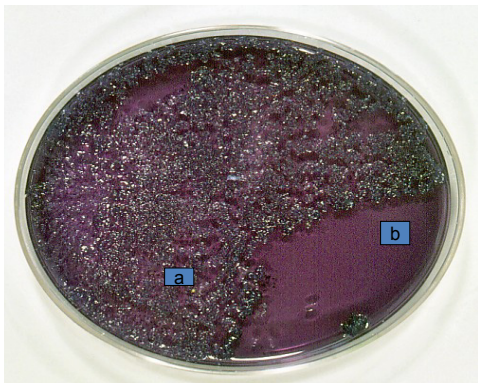
### EN-like lesion and its histology



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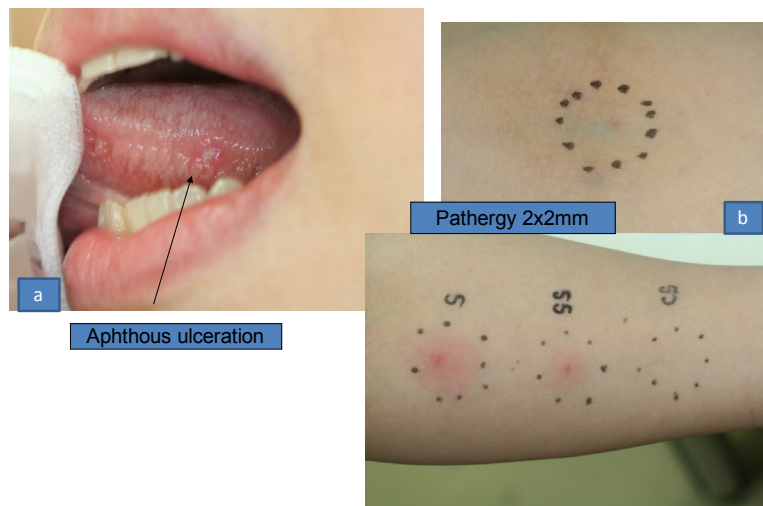
**Figure 2** a. EN-like eruption of a BD patient. b. A biopsy specimen showed vasculitis infiltrated by lymphoid cells and neutrophils in the dermis (HE stain,  $\times 400$ ). c. The vascular lesion was stained by anti-streptococcal antibody (Immunofluorescence,  $\times 400$ ).

### Streptococci from saliva of a BD patient in SM agar



**Figure 3** When saliva from a BD patient was incubated in Salivarius and Mitis (SM) agar, oral streptococci are limited grown in a few days. In a. area, streptococcal colonies from crude saliva were grown and in b area, no bacterial colonies was recognized from the saliva sterilized through the micro-filter with 0.2  $\mu$ pores.

### Incomplete BD female patient (35 F, YS)



**Figure 4** A clinically typical and active case of 35 year-old female BD patient classified as "Incomplete type" by Japanese Classification (a). Although 2 mm erythema-reaction appeared by "Pathergy test" (b), more than 20mm diameter erythematous reaction was recognized 24-48 hours after self salivary prick (Salivary prick test). Also, by the sterilized saliva, more than 10mm erythema reaction was observed in this case (c).



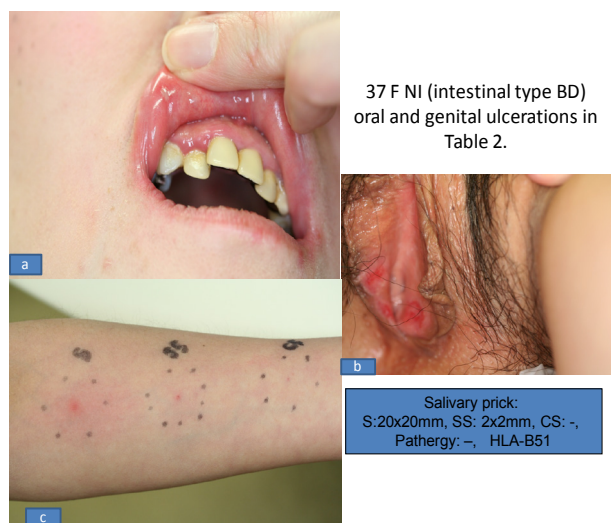
**Genital ulcer:** The clinical features of genital ulceration are generally shaped as similar to oral aphthous ulceration of BD patients (Figure 5). A few cases of young female are suddenly attacked by genital ulceration as the initial BD symptom clinically like Lipschutz genital ulceration<sup>[32]</sup>, which is supposed to be due to Epstein-Barr viral (EBV) infection<sup>[33,34]</sup>. However, EBV was not detected from the lesion as our case listed in Table 1. About more than 50% of BD patients are found to be associated with genital ulceration (female: 55.5%, male: 58.7%), that is, ulcers occur on vulva (66.1%), vaginal mucosa (35.7%), anus (9.6%), cervix (4.1) and groin area (0.8%) in female and on the penis (46.5%), scrotum (38.5%), anus (9.2%) and groin area (5.0%) in male patients<sup>[2,4]</sup>.

**EN-like eruption of BD patients and non BD-EN:** More than 50% of BD patients are reported to be associated with EN-like eruption on the lower legs<sup>[2-4]</sup>, which looks smaller to EN of non-BD patients (Figure 2a). Generally, the histology is “vascular reaction” infiltrated by mainly lymphoid mononuclear cells, so-called “lymphocytic vasculitis”, and septal panniculitis in the subcutaneous fatty tissue. In acute and active phase of BD patients, however, vasculitis surrounded by neutrophils is able to be recognized in a few days after the occurrence. Although it is difficult to differentiate BD-EN like eruption from non-BD EN, features of venous thrombosis features are sometimes found in active BD-EN<sup>[35]</sup>. Immunofluorescence revealed deposits of IgA, IgM, C3 and streptococcal related materials by anti-streptococcal antibody in the vascular walls (Figure 2c,d)<sup>[12,17,31]</sup>. On the other hand, the streptococcal related materials could not be detected in our cases with non-BD EN tissues. The findings suggest that streptococcal antigens might be playing an important role in the BD symptoms as the triggering extrinsic factor<sup>[11,12,17,31]</sup>. It is of interest that GroEL of *S. sanguinis* and human heterogenous nuclear ribonucleoprotein (hnRNP) A2/B1 were expressed on the vascular walls<sup>[36,37]</sup>. However, the causation of non-BD EN is also unknown, but the majority of EN patients have evidence of recent streptococcal infection or have no identifiable causes<sup>[38,39]</sup>.

### PATHERGY TEST AND ORAL STREPTOCOCCI

It is not difficult to make a diagnosis for BD except for the atypical cases without the main muco-cutaneous symptoms including RAS. Pathergy reaction, which is a non-specific cutaneous hypersensitive response showing around 2 mm pustule 24-48 h after pricking with 20 G syringe needle, has been thought to be helpful to diagnose BD, because the phenomenon has been believed as a unique feature of BD described by International Study Group of BD<sup>[40]</sup>. Histology and immunohistology of the “Pathergy reaction” is similar to those of EN-like eruption from BD patients<sup>[41,42]</sup>. However, recently the reactivity by Pathergy test became chronologically lower to less than 40% of BD patients seen in 2007s, though the response was seen in more than 70% of the patients in 1970s. The patients associated with HLA-B51 were thought to show stronger skin reaction by Pathergy test<sup>[43]</sup>, but its diagnostic value is different from the prevalence in the countries<sup>[44-46]</sup>. In our cases, only one of 22 cases showed a 2 mm pustule 48 h after pricking with 20 G syringe needle, which Pathergy reactivity was less than 5% (Figure 3b). It is of interest that the surgical cleaning of the forearm before needle prick reduced its reactivity<sup>[47]</sup>, suggesting that the “Pathergy reaction” might be a response to some bacteria living on the surface of the skin. Then, instead of “Pathergy test”, we tried to prick with self-saliva including oral *streptococci* (Salivary prick test) (Figure 3), to the forearm of BD patients using Lancetter with tiny stick (OY ALGO AB Espoo/ Esbo, Sweden), because the patients have hypersensitivity to

streptococci. The results revealed more than 90% of 22 BD patients showed larger than 5 mm erythematous reaction with spot pustule by Salivary prick test (Figures 4 and 5, Tables 1 and 2). The histology of the positive site was basically similar to that of BD-EN-like lesions. Non-BD RAS and Lipschutz genital ulcer patients showed weaker reaction than those of BD patients, suggesting some possibilities to differentiate from each other and also correlation with *streptococci* in the pathogenesis. No reaction and/or tiny spot were seen by the prick with microfilter-sterilized saliva and saline in BD patients and the disease controls including patients with viral aphtha and non-BD EN and healthy persons (Table 1). Regarding non-BD RAS, the results also suggest that oral *streptococci* are playing an important role in the pathogenesis of RAS of BD patients, although there are many



**Figure 5** A 37 year-old female active BD patient classified as incomplete type and associated with HLA-B51. a. Aphthous ulceration of the r-upper lip membrane. b. genital ulceration, c. The Salivary prick test revealed 20 × 20 mm, SS: 5 × 5 mm erythematous reactions and CS: negative, though “Pathergy test” was negative

**Table 1** Self-salivary prick test in patients with aphthous ulceration and controls.

Age, Sex	Prick test: S	Small pustule	SS	CS	Pathergy
Japanese classification	Patients (initials)	(after 48 h)			(22 G syringe)
Neuro BD 55 M YY	11 × 15 mm	+	nd	-	-
Incomplete 33 F AT*	22 × 22	-	-	-	-
26 F MN*	10 × 10	+	+ dot	-	dot
27 M TG	11 × 12	+	+ dot	-	dot
47 M YT	10 × 13	+	+ dot	-	dot
36 F MY*	5 × 10	-	nd	-	-
46 M KH	10 × 10	-	-	-	-
17 F YT	5 × 5	-	-	-	-
35 F YS	23 × 26	+	12×14 mm	-	2 × 2mm
Complete 23 M OO*	10 × 10	+	-	-	-
50M HS	13 × 14	-	-	-	-
Recurrent aphthous stomatitis (RAS)					
24 F YN	8 × 10 mm	-	-	-	-
28 F YS	8 × 4	-	-	-	-
32 F YN	-	-	nd	-	-
29 M ON	-	-	nd	-	-
28 F MS	3 × 5	-	-	-	-
10 F AY**	6 × 7	-	2 × 2	-	-
Disease controls					
non BD EN 39 F KY	-	-	nd	-	-
61 F MF	-	-	-	-	-
Lipschutz GU 16 F NH***	4 × 4	-	2 × 2	-	-
viral aphthosis (3)	-	-	nd	-	-
Healthy controls (8)	-	-	-	-	-

BD: Behcet’s disease’ EN: erythema nodosum; F: female; M: male; dot: small spot; +: positive, -: negative; S: prick with self-saliva; SS: prick with sterilized self-saliva; CS: prick with control saline; nd: not done; The clinical type of BD is followed by the Japanese BD classification. \* Same cases in Table 2.

**Table 2** Self-salivary prick test in BD patients with or without HLAB51.

Type of BD (Japanese classification)	Patients(initials)	Prick test (mm)			HLA-B51
		S	SS	CS	
Complete type	23 M OO*	10	-	+	(B51)
Intestinal type	37 F NI	20	2	-	+ (B51,52)
Incomplete type	40 M HG	10	7	-	+ (B51,01,01)
	31 F MA	30	7	-	+ (B51,40)
Incomplete type	42 F MK	7	4	-	+(B51,46,DR4,8)
	34 F AN	26	5	-	- (B35,48)
	36 F MY*	10	-	-	- (B44,03,01)
	33 F YK	10	-	-	-
	33 F AT*	22	-	-	- (B40,48)
	26 F MN*	10	-	-	-
	35 M YI	10	nd	-	- (B15,35)
	37 F HT	4	-	-	- (B40,44)
	36 M MK	4	3	-	- (B35,44)
	35 M KF	7	2	-	- (B46,54)
35 F YO	14	-	-	- (B07, 02, 01)	

There are some reports that BD patients associated with HLA-B51 show much stronger reaction to "Pathergy test". Although the patients with HLA-B51 showed relatively stronger skin reactions, the reactive severity seemed to be correlated with the disease severities. Five out of 15 patients (33.3%) were HLA-B51 possessor.

studies, still no clear causation is present<sup>[50,51]</sup>. The Salivary prick test is considerable to make a differentiation of BD from non-BD disorders with similar symptoms. The case with Lipschutz genital ulceration showed a weak skin reaction to self-saliva (Figure 6a, b, Table 1)<sup>[49]</sup>.

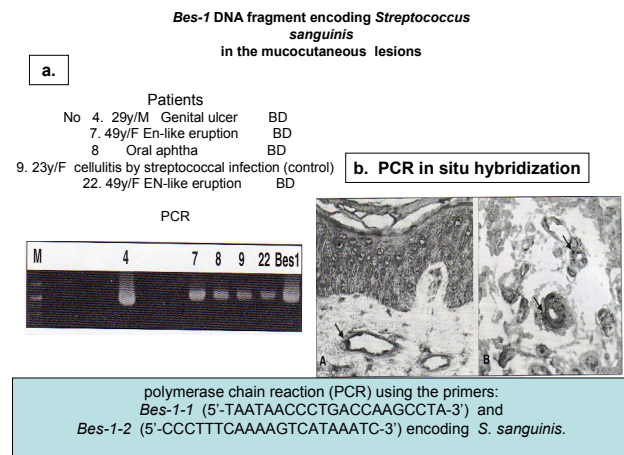
## HLA GENOTYPING AND STREPTOCOCCAL INFECTION

HLA-B51 is supposed to be a highly associated with BD patients as the genetic marker even in many different ethnic groups including European, Mediterranean and Asian people. BD has several unique epidemiologic features which seem to go from Southern Europe to Japan along "the old Silk Road", as mentioned previously<sup>[5,7,8,52,53]</sup>. The appearance of BD lesions is not directly correlated with HLA-B51 in the immunologic background of the patients, but it was found that HLA-B51-restricted cytotoxic T lymphocytes (CTLs) played some roles in correlation with the stressed target tissues expressing major histocompatibility complex class I chain-related gene A (MICA) in BD pathogenesis. When the transmembrane-MICA is preferentially expressed on epithelial and endothelial cells by stress, they seem to be the candidates for the HLA-B51-restricted CTLs response. The endothelium are also considered to be the ligand for activating natural killer (NK) cells with NKG2D molecule and CD8<sup>+</sup> T cells as CTLs<sup>[54,55]</sup>. Regarding NK cell activation, inhibitory CD34/NKG2A and activating CD94/NKG2C molecules are alternatively expressed on NK, CD4<sup>+</sup>CD8<sup>+</sup> T cells, as indicating an imbalance in cytotoxic activity in BD patients<sup>[56]</sup>. However, the function of NK cells is supposed to be down-regulated in the active stage and to be up-regulated in the remission of BD patients<sup>[57]</sup>. On the other hand, the expressive CD4<sup>+</sup>T cells activated by inflammatory cytokines including interferon (IFN)- $\gamma$ , IL-12, IL-23, etc. might be altered to Th17 cells, which release IL-17 in the BD lesions, as seen in autoimmune disorders<sup>[58]</sup>.

HSP-65/60 derived from microorganism including *S. sanguinis* and damaged human tissues, which is actually detectable in the oral mucosal and skin lesions of BD patients, also becomes a stress-inducible factor in connection with MICA\*009 expression<sup>[23,24]</sup>. Generally, it has been reported that antigen presenting cells (APCs) expressing IL-12 are thought to be activated in BD patients with HLA-B51 in the active stage, as seen in transgenic mice<sup>[52]</sup>. However,



**Figure 6** a. A case of Lipschutz genital ulceration of 17 years old female. No EBV titer was detected. b. These are cutaneous reactions by Salivary prick test. Crude salivary prick (S) showed around 4mm in diameter. The skin reaction was suggesting streptococcal hypersensitivity



**Figure 7** *Bes-1* gene expression in the mucocutaneous lesions of patients with BD. a. Three of 11 BD patients were positive for *Bes-1* DNA in the lesions including aphthous and genital ulcerations and erythema nodosum (EN)-like eruption by amplified polymerase chain reaction (PCR) using the primers: *Bes-1-1* (5'-TAATAACCCTGACCAAGCCTA-3') and *Bes-1-2* (5'-CCCTTTCAAAAGTCATAAATC-3') encoding *S. sanguinis*. b. In these positive lesions, *Bes-1* DNA was also detected in the cytoplasm of monocytes adhering to the vascular walls and infiltrated around the vessels by PCR in situ hybridization.

we have obtained interesting results that PBMCs from BD patients without HLA-B51 gene can be significantly stimulated by *S. sanguinis* antigen in the expression of IL-12p40 mRNA and that its protein level was also increased in connection with IL-12p70 (p35 and p40 subunits) rather than those of the patients with HLA-B51<sup>[59]</sup>. The antibacterial host response by T cell type immunity mediated by IL-12 is suggested to be much stronger in HLA-B51-negative BD patients in vitro experiment. In our cases, about 33% of the patients were associated with HLA-B51 (Table 2) and the severity of the Salivary prick test might be correlated with the disease activity in BD patients, though Pathergy test was reported to be stronger than those of patients with HLA-B51<sup>[45,49,60]</sup>.

## HYPERSENSITIVITY AGAINST S. SANGUINIS:

Generally, the oral health is impaired in BD patients with the disease severity<sup>[11-13,15,16]</sup>. The antibodies against *S. sanguinis* showed cross reactivity with the synthetic peptides of HSP-65 derived from the

bacteria<sup>[61,62]</sup> and delayed type cutaneous hypersensitivity reactions against streptococcal antigens were also seen in BD patients. Actually BD symptoms were provoked by the antigens and aphthous ulceration can be also induced by a prick with streptococcal antigen on the oral mucous membrane of a BD patient<sup>[11,12,17,18]</sup>, which is so-called “oral bacterial allergic reaction”. Isogai *et al*<sup>[62]</sup> demonstrated that the symptoms mimicking BD appeared in germ-free mice when *S. sanguinis* from BD patients was inoculated into their oral tissue damaged by heat shock and/or mechanical stress. This report suggests that the immunization with *S. sanguinis* through the oral membrane route elicits BD-like symptoms in the animal model. We tried to find the presence of *Bes-1* gene by polymerase chain reaction (PCR) in BD lesions using 2 distinct primer sets (peptides, 229-243 and 373-385) encoding *S. sanguinis* (serotype KTH-1) prepared by Yoshikawa *et al*<sup>[63]</sup>. *Bes-1* DNA was present in various muco-cutaneous lesions including oral and genital ulcerations and EN-like lesions. PCR-in situ hybridization revealed *Bes-1* DNA expression in the cytoplasm of inflammatory infiltrated monocytes adhering the vascular walls in muco-cutaneous lesions (Figure 7)<sup>[64]</sup>. In contrast, we failed to detect DNAs of herpes simplex virus (HSV)-1, HSV-2, cytomegalovirus, human herpes virus (HHV)-6 and HHV-7 in the lesions by PCR<sup>[65]</sup>, although it is reported that the animal models infected by HSV demonstrated to mimic BD like symptoms<sup>[66]</sup>.

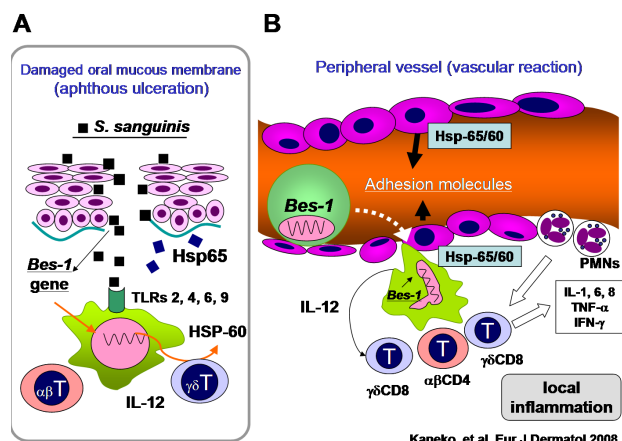
Interestingly, the amino acid sequence of the peptides of *Bes-1* (229-243 and 373-385) shows more than 60% similarity to the human intraocular ganglion peptide, *Brn-3b* which is a subfamily of POU (pit-Oct Unc) domain factors containing *Brn-3a* and *Brn-3c*<sup>[67]</sup>. The peptide of *Bes-1* (229-243) was also found to be correlated with the peptide of HSP-60 (336-351)<sup>[61]</sup>. These results suggest that *Bes-1* derived from oral *S. sanguinis* might be an inducer for the possible retinal and neural involvement in BD patients.

## HSP-65 DERIVED FROM MICROORGANISM AND HUMAN HSP-60

HSPs, which scavenge denatured intracellular proteins, are supposed to be induced by microorganisms and mammalian tissues under a variety of stressful condition<sup>[68]</sup> and they may be involved in the pathogenesis of some autoimmune diseases<sup>[69]</sup>. The serum levels of IgA to mycobacterial HSP-65, which cross-reacts with selected strains of *S. sanguinis*, are significantly increased and HSP-60 was also detected in various lesions in BD patients<sup>[70,71]</sup>. On the other hand, 4 peptides of HSP-65 (111-125, 154-172, 219-233 and 311-326) derived from *S. sanguinis*, which are 50-80% homology to the counterpart human HSP-60, were recognized as immuno-dominant agents for T and B cell responses<sup>[24,72-74]</sup>. The 4 peptides of HSP-65 were also shown to significantly stimulate and undergo CD4<sup>+</sup> and CD8<sup>+</sup> T cell apoptosis in PBMCs from BD patients and HSP-60 also seemed to stimulate them<sup>[71]</sup>. On the contrary, the other two peptides of HSP-65 (21-35 and 401-415) corresponding to human HSP-60 (425-441) are reported not to stimulate PBMCs from BD patients and healthy individuals<sup>[68]</sup>. The peptide of HSP-60 (336-351) was also identified to be highly homologous to T cell epitope<sup>[68,70-76]</sup>. Whole HSP-60 is, however, suspected to induce vascular endothelial growth factor (VEGF) which activates, impairs and propagates the vascular endothelial cells<sup>[76]</sup>. It may also lead to thrombophlebitis and vasculitis by damaging endothelial cells in BD patients, although non-BD EN does not seem to be associated with thrombophlebitis<sup>[78,79]</sup>.

It is of interest that the peptide of HSP-60 (336-351) linked to recombinant cholera toxin B subunit (rCTB) reduced the uveitis induced by whole HSP-60, although the peptide without the

## Aphthous ulceration and systemic symptoms in BD patients



**Figure 8** A hypothesis showing the relationship between RAS and the systemic lesions in BD patients (97,98). a. The antigen presenting cells (APCs) (macrophages and/or dendritic cells) are immunized by streptococcal related antigens (*Bes-1*) including *S. sanguinis*, through toll-like receptors (TLRs) expressing 2,4,6, and 9, as “innate immune mechanism”. The APCs expressing *Bes-1* gene in the oral lesion might be carried through vascular ways to the peripheral regions. b. The APCs bond at the impaired and/or MICA (major histocompatibility complex class 1- related gene A) gene expressing endothelial cells of the peripheral vascular wall. Heat shock proteins (HSPs) 65/60 will be induced and the immunological reactions may be caused as BD lesions.

adjuvant is reported to induce uveitis in Lewis strain rats<sup>[80-82]</sup>. A therapeutic trial by the peptide conjugated with rCTB was performed to BD patients with recurrent uveitis. The successful results were obtained to show that 5 of 8 patients had no relapse of uveitis, and that 2 of the remaining 3 patients had improved recurrent oral ulceration, folliculitis, EN-like eruptions and genital ulcers without any side-effects. In those patients with uveitis and extra-articular manifestations, a lack of the peptide-specific CD4<sup>+</sup> T cell population, a decrease in expression of Th1 type cells (CCR5, CXCR3) and a reduction of IFN- $\gamma$ , TNF- $\alpha$  and CCR7<sup>+</sup> T cells were observed in comparison to BD patients with relapse of disease<sup>[82]</sup>. The HSPs presented by APCs can directly stimulate  $\alpha\beta$ <sup>+</sup>T and  $\gamma\delta$ <sup>+</sup> T cells which play important roles in the oral mucosal immunity as the first defense against microorganisms. It is thought that  $V\gamma 982$ <sup>+</sup> T cells, a major subset of  $\gamma\delta$ <sup>+</sup> T cells, which recognize antigens in the innate and adaptive immune responses, were influenced by secreting IFN- $\gamma$ . The  $\gamma\delta$ <sup>+</sup> T cells expressing CD29 and CD69 produce IFN- $\gamma$  and TNF- $\alpha$  from stimulation by HSP-65/60 in the lesions of BD patients with active disease<sup>[83]</sup>. In the active stage of BD patients, IL-12 as a sign of Th1 type reaction, is also produced and advanced the symptoms. It is interest that the gene polymorphism in the promoter region regarding a 4 bp insertion within IL-12p40 (IL-12B) was significantly higher in the HLA-B51 negative BD patients. The expression of IL-12B mRNA and protein levels were also significantly increased in PBMCs from BD patients without HLA-B51 by stimulation with *S. sanguinis* antigen<sup>[58]</sup>. The expression of IL-23, which is composed of a shared p40 subunit of IL-12 and p19 subunit of IL-23 was also increased in EN-like lesions of BD patients<sup>[84,85]</sup>.

The therapeutic approaches using the peptide of HSP (336-351) linked to rCTB were applied for BD patients with advanced uveitis, as “oral toleration” demonstrated by Stanford *et al*<sup>[82]</sup>. In order to understand the suppressive mechanisms of the cytokine production in PBMCs from active BD patients, we tried to find the binding sites of the peptides on monocytes by cDNA chips (Gene Chip; Human Genome) using NOMO-1 cells (human macrophage cell line)



activated by *S. sanguinis* antigen. Although the expression of IL-8, IL-16, IL-13R and IL-17R was decreased after incubation with LO1 and UK, respectively, LO2 did not decrease IL-8 production. CD58 (lymphocyte function-associated antigen-3) molecule and/or FK506 binding protein were highly expressed on the cell membrane after application of LO1 and UK<sup>[86,87]</sup>.

## TOLL-LIKE RECEPTOR (TLR) EXPRESSION

Regarding the recognition system for the microorganism antigens in humans, 10 numbers of TLR families are supposed to act as innate immune receptors by binding of particular structures present on bacteria, viruses, fungi, etc.<sup>[88]</sup>. TLR-3 [ds RNA] and TLR-6 [mycoplasma, staphylococci, etc.] are also reported to be enhanced in expression on neutrophils and monocytes of BD patients, when stimulated by HSP-60 and *S. sanguinis* antigen<sup>[89]</sup>. In RAS lesion of BD patients, expression of TLR-9 [unmethylated CpG DNA, bacteria and virus] has been also found<sup>[90]</sup>. These findings suggest that innate immune system contributes the acquisition of hypersensitivity against oral *streptococci* in the pathogenesis of BD as the extrinsic factor.

## COMPLEMENT SYSTEM

Deposits of complement C3 with immunoglobulins are frequently detectable at the vascular involvement by immunofluorescent techniques in BD patients<sup>[17,31,79]</sup> and the titer of serum complement is generally high in the inactive stage. However, the levels of mannose-binding lectin (MBL) pathway are generally decreased in the patients<sup>[91]</sup>. The MBL pathway is considered to play an important role in the innate immunity. Ficolin (FCN) is a soluble protein that binds to carbohydrate on the microbial cells and 3 different types of FCN are detected. FCN 1 and 2 genes are located in the chromosome 9q34 and FCN3 gene is assigned to chromosome 1. FCN 2 binds to lipoteichoic acid on the cell wall constituent in all Gram-positive bacteria and activate immune cells to produce proinflammatory cytokines<sup>[92]</sup>. We have found that novel FCN 2 gene single nucleotide polymorphisms (SNPs) are identified in the promoter regions as well as in the exon regions. The MBL genetic polymorphisms might be involved in immune responses to streptococcus infections in BD patients, because the relationship between MBL gene mutations and microbiological factors were suspected in the lesional immune reaction of BD patients<sup>[93]</sup>. Although no significant difference was present in the genotype allele frequencies of MBL gene SNPs between BD patients and healthy controls, the allele frequencies of FCN2 gene SNPs were significantly recognized in the promoter regions (-557 and -64 sites) among HLA-B51 positive BD patients<sup>[94]</sup>. The findings suggest the possibility that FCN gene of the MBL pathway in complement system contributes to the innate immunity in BD patients.

## RAS AND SYSTEMIC SYMPTOMS

BD symptoms are characterized by vascular involvements histologically showing swollen endothelial cells of the micro-veins infiltrated by inflammatory monocytes with a few neutrophils, so-called “vascular reaction” seen in EN-like eruption and other lesions<sup>[17,42,78,79]</sup>. The strong hypersensitivity reaction against *S. sanguinis* agents carried by APCs can be suspected in the pathogenesis of BD which may be one of the extrinsic triggering factors<sup>[11,12,17,18,20]</sup>. Regarding the treatment, low dose administration of minocycline is clinically effective for BD patients, because minocycline is experimentally administered not only to decreases

a growth of oral *S. sanguinis* but also works to suppress IL-1 $\beta$  and IL-6 production from T cells inflamed. Actually, we recognized clinically effective for RAS, acne-like eruption and EN-like lesion in BD patients<sup>[12]</sup>. Other studies also showed that combination therapy, colchicine and benzathine penicillin, was effective to suppress BD symptoms compared to colchicine monotherapy<sup>[95,96]</sup>. The oral infectious agents suggest the hypothesis that after *Bes-1* gene derived from *streptococci* taken in the cytoplasm of APCs through the TLRs in RAS lesion of BD patients, the APCs carrying the streptococcal antigen produce HSP-65 in the peripheral vascular lesions. The APCs impair MICA expressed endothelium of the vessels in correlation with HSP-65/60, VEGF, adhesion molecules, etc. BD lesions will be induced by the “vascular reaction” and/or “lymphocytic vasculitis” as the immunological reactions due to the APCs expressing *S. sanguinis* antigen<sup>[97,98]</sup> (Figure 8).

## THERAPY

In order to treat for BD patients, we should know about the clinical manifestations and pathogenesis, as described above. It is important to analyse clinical metabolic biomarkers of inflammation in the advanced systemic symptoms of BD including involvements of ocular, vascular, neurosystem and gastrointestinal organs. However, though the therapy for the muco-cutaneous symptoms such as RAS, genital ulceration and acne-like eruption is centered on the topical measures, low dose of minocycline capsule (50-100 mg/day) for long time treatment is effective not only for the clinical symptoms, but also inflammatory cytokine production from activated lymphoid cells, as described previously<sup>[12]</sup>. And administration of colchicine (0.5-1.0 mg) can also manage the inflammation of EN-like symptoms and the joint involvements<sup>[99]</sup>. As to the immune reduction, azathiopurine, cyclosporine and corticosteroids are used in cases with severe resistant muco-cutaneous and articular manifestations of BD. To date, in the point of immunological mediators correlated to the systemic involvements, some biological antibodies, as infliximab, adalimumab, etc., are applied for BD patients<sup>[100]</sup>.

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## CONFLICT OF INTERESTS

The authors declare that they do not have conflict of interests.

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