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Neutrophil and lymphocyte responses to oral streptococcus in Adamantiades-Behçet's disease

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Abstract

Immune reactions against microorganisms play an important pathogenic role in Adamantiades-Behçet's disease (ABD). We had previously obtained *Streptococcus sanguinis* (strain BD113-20) isolated from the oral cavity of patients with ABD. To
5 investigate the pathogenesis of this isolate, we examined neutrophil reactions and level of cytokine production by lymphocytes after stimulation with the strain. The reactions of neutrophils were examined by chemiluminescence assay using whole blood. The amounts of interferon gamma (IFN- γ) and interleukin (IL)-4, IL-8, IL-10, and IL-12 produced by peripheral blood mononuclear cells (PBMCs) were measured by ELISA.
10 Strain BD113-20 activated neutrophils from patients with ABD and healthy volunteers, and, in addition it increased IFN- γ production by lymphocytes. Lymphocyte from the patients with ABD showed a dominant T helper 1 (Th-1) immune response. Results indicated that both bacterial stimulation and host hypersensitivity might be involved in the symptoms and pathogenesis of ABD.

15 Key words

Adamantiades-Behçet's disease, *Streptococcus sanguinis*, neutrophil, chemiluminescence, IL-8, T helper-1, IFN- γ IL-12

1. Introduction

Adamantiades-Behçet's disease (ABD) is a multi-system disorder that presents with recurrent oral and genital ulceration as well as uveitis, leading sometimes to blindness. Although the etiology and pathogenesis of this disease remain obscure, 5 different bacteria and viruses have been suggested as causative factors. Several investigators have reported that streptococcal infection is related to the onset of ABD. Patients with ABD have shown significantly higher incidences of tonsillitis and dental caries, probably due to streptococci, and the lymphocytes obtained from such patients were sensitive to streptococcal antigens [1, 2]. Additionally, patients with ABD showed 10 hypersensitivity in skin tests to streptococcal antigens [3]. We have previously shown that the proportion of *Streptococcus sanguinis* (strain BD113-20 isolated from a patient with ABD) in the oral flora of patients with ABD was significantly higher than in controls [4], and the serum anti-BD113-20 titers of the patients were also higher than titers to standard strains [5]. In another study, patients' sera did not neutralize bacterial 15 IgA protease purified from BD113-20, demonstrating that humoral immunity against oral streptococci in patients with ABD was not sufficient to exclude the oral bacteria [6]. Taken together, these results suggest that both host immunity and oral bacteria are involved in the pathogenesis of ABD.

In this study, we demonstrate the neutrophil and lymphocyte reactions against *S.* 20 *sanguinis* BD113-20 isolated from patients with ABD. Neutrophil reactions were examined by chemiluminescence (CL) assay using whole blood stimulated with streptococcal antigens and zymozan. Peripheral blood mononuclear cells (PBMCs) were also stimulated with streptococcal antigens, and their T helper 1- and 2-associated cytokines were investigated.

2. Materials and Methods

2.1. Patients

Nineteen patients with ABD (8 men and 11 women; aged 32 to 79, mean 55.8 years), diagnosed according to the international study group criteria were studied.

5 Historical information of 19 patients with ABD is shown in Table 1. Clinical information at the time of drawing blood is also shown in Table 2. Twenty-one healthy volunteers (11 men and 10 women; aged 21 to 41, mean 30.8 years) were used as controls. Heparinized blood was obtained of which a portion was used for neutrophil examination within 2 hours. The remaining heparinized peripheral blood was layered on
10 a Ficoll-Paque density gradient solution and centrifuged (3000xg, 30 min). The upper layer, containing plasma and platelets, was removed, and the buffy coat, the rich fraction in peripheral mononuclear cells (PBMCs) collected. The PBMCs were washed twice with phosphate buffered saline (PBS, pH 7.2) and cultured in RPMI1640 medium containing 10% fetal calf serum (FCS).

15 2.2. Bacterial strains

Streptococcus sanguinis strain BD113-20, isolated from the mouths of patients with ABD, was employed. *Streptococcus pyogenes* (WHO-T type 12), *Streptococcus salivarius* (ATCC 7073), and *Streptococcus sanguinis* (ATCC 10556) were also used as control strains. All strains were cultured in Todd-Hewitt broth (Difco, Detroit, MI) at
20 37C for 18 hr. The cells were harvested by centrifugation (6,000xg, 20 min, 4 C) and washed three times with 10 mM PBS. The whole cells were disrupted with a Branson cell homogenizer (Branson model 250 sonifier, Branson Ultrasonics, Danbury, CN), and the unbroken cells were removed by centrifugation (6,000xg, 20 min, 4 C). The supernatants were again centrifuged at high speed (20,000xg, 30 min, 4 C), and the

resulting supernatants were designated as the 20S antigen.

2.3. Chemiluminescence (CL) assay

Neutrophil activity was measured using a CL assay. Heparinized blood (100 μ l) was suspended in 400 μ l of Hanks solution with 20 mM HEPES and 10mM CaCl₂ (pH 7.4, HEPES-Hanks). The blood in the HEPES-Hanks solution was added to 10 μ l of luminol solution (2 mg ml⁻¹), and then stimulated with 50 μ l of streptococcal antigens (10mg ml⁻¹) or non-opsonized zymozan (5 mg ml⁻¹). The antigens' CL was immediately measured with a luminometer (Biolumat LB9505, Berthord, Germany).

2.4. Measuring Cytokine production levels

PBMCs (1x10⁶ cells ml⁻¹) were washed three times in RPMI 1640 by centrifugation. Aliquot of 500 μ l of cell suspensions were cultured in a 48 well plate stimulated with the bacterial antigen of *S. sanguinis* (ATCC10556), BD113-20 (10 μ g ml⁻¹), or concanavalin A (5 μ g ml⁻¹: conA). Culture supernatants were collected at one week, and stored at -80 C. IFN- γ , IL-4, IL-8, IL-10 and IL-12 levels were measured using an ELISA kit (Biosource, Camarillo,CA) according to the manufacturer's instructions.

2.5. Statistical analysis

Results were expressed as means + standard error (SE). Data were compared using a Student's t-test, and statistical differences were considered significant if p values were <0.05.

3. Results

3.1. Neutrophil activation

BD113-20 was isolated from a patient with ABD and used to assess stimulation of neutrophils in a CL assay. The stimulation by BD113-20 was significantly greater
5 ($p < 0.05$) in both ABD and control subjects than was observed for other Streptococcus
antigens or by zymosan (Fig. 1). CL in patients with ABD was also activated by the *S.*
sanguinis type-strain (ATCC10556). However, on stimulation with *S. pyogenes*, CL did
not differ between patients and controls. In the healthy volunteers, stimulation with *S.*
salivarius induced strong CL activity. These results indicate that in the ability to activate
10 neutrophils, BD113-20 may differ from other oral streptococci.

Stimulation with strain BD113-20 induced high levels of IL-8 production by
PBMCs in both the patients ($867 \pm 55 \text{ ng ml}^{-1}$) and the controls ($599 \pm 82 \text{ ng ml}^{-1}$) (Fig. 2).
Also, the level of production was significantly ($p = 0.027$) higher in the patients with
ABD than controls. The *S. sanguinis* type-strain did not induce IL-8 production either in
15 patients with ABD or in controls. Infection with BD113-20 in patients with ABD may
enhance neutrophil activation.

3.2. Levels of Th-1/2-associating cytokines

Stimulation with BD113-20 induced high levels of IFN- γ production from
PBMCs of patients with ABD ($2254 \pm 238 \text{ pg ml}^{-1}$) and controls ($2301 \pm 399 \text{ pg ml}^{-1}$) (Fig.
20 3). The type-strain of *S. sanguinis* (ATCC10556) induced IFN- γ ($1075 \pm 249 \text{ pg ml}^{-1}$)
production by PBMCs from the patients with ABD, however little IFN- γ ($125 \pm 69 \text{ pg ml}^{-1}$)
was produced by lymphocytes from controls.

The type strain of *S. sanguinis* (ATCC10556) and BD113-20 induced a high
level of production of IL-12 (*S. sanguinis* (ATCC10556) stimulation; $1975 \pm 410 \text{ pg ml}^{-1}$,

BD113-20 stimulation; 2540+430 pg ml⁻¹) by PBMCs of the patients (Fig. 4). On stimulation with any antigen, IL-12 production from PBMCs from patients with ABD was significantly increased compared to that by PBMCs of controls.

Levels of Th-2 associated cytokines (IL-4 and IL-10) were low. Specifically, little IL-4 was detected in PBMCs of patients with ABD or controls (data not shown). Stimulation with the *S. sanguinis* type-strain (ATCC10556) induced significant IL-10 production (130+34 pg ml⁻¹) from PBMCs in patients with ABD (Fig. 5), while conA stimulation did not enhance IL-10 secretion from PBMCs. On the other hand, the healthy volunteers' PBMCs produced IL-10 following stimulation with BD113-20 (95+33 pg ml⁻¹) and conA (72+33 pg ml⁻¹). For PBMCs from controls, the IL-10 level, without stimulation, was below the detection limit. These results indicate that patients with ABD have a Th-1 dominant immunity, and infection with BD113-20 might enhance the Th-1 immune reaction.

3.3 Disease activity

The mean age when first symptoms arose in the patients was 36.7 years old, and 12 of 19 patients (63.5%) had developed aphthous stomatitis as a first symptom (Table 1). Disease activity of each patient is shown in Table 2. The CL and production of cytokines were not associated with active or inactive symptoms (Data not shown).

4. Discussion

Immunopathogenic mechanisms are closely associated with the etiology of ABD. Both microbial antigens and host immune responses may contribute to this multi-systemic disorder, presenting various symptoms [7]. We have previously reported that *S. sanguinis* BD113-20 isolated from a patient with ABD, may be an important bacterium stimulating host immunity [4, 5, 6]. In this study, neutrophil reactions and T helper (Th-) 1 or 2 immunity-associated lymphokines were examined using soluble antigens of the strain.

Neutrophil activation is involved in the pathogenesis of ABD, as evidenced by the lesions that are characteristic of neutrophil infiltration of the pustular folliculitis and hypopyon [8]. It has been reported that neutrophil reactions were associated with some viridans *Streptococcus* or group B *Streptococcus* [9, 10]. In this study, we found that BD113-20 significantly enhanced CL activity in neutrophils, and may be different from other oral streptococci. In addition, as a host factor, IL-8 was produced at high levels by lymphocytes of patients with ABD than healthy volunteers. High levels of expression of IL-8 mRNA in patients with ABD have also been reported [11, 12], and IL-8 seems to be a reliable marker for disease activity [13]. Neutrophil activation may be promoted by the IL-8 released from lymphocytes and/or endothelial cells of patients with ABD. *Streptococcus* exists as oral flora in patients with ABD, whose symptoms may often be caused by dental treatment or aphthous stomatitis.

Throughout life, viridans streptococci comprise a large proportion of the commensal microbiota in the oral cavity and pharynx [14], and different species of viridans streptococci are known to colonize distinct habitats within the mouth [15]. Uncommon streptococcus strains, such as strain BD113-20, were increased in oral flora

of patients with ABD [16], and were not eradicated by IgA mucosal immunity [6]. Both persistent infection by the neutrophil-activating *Streptococcus* and the lymphocyte responses are presumed to play an important role in the symptoms of ABD.

Long-term bacterial stimulation will induce adaptive immunity in patients with
5 ABD. T-cell activation, both in the peripheral blood and in tissue specimens, occurs in
ABD. The CD4-positive cells are divided into Th-1 and Th-2 cells. The differential
effects of Th-1 and Th-2 populations are mediated through their characteristic cytokines.
IL-12 is produced by macrophages and is a potent inducer of IFN- γ production by Th-1
cells and natural killer cells. IL-12, in concert with the presentation of antigens by
10 dendritic APC, is believed to promote the induction of CD4 positive T_{DTH} cells and T_{CTL}
cells, thus directing the immune response to cell-mediated immunity. IL-12 production
was significantly increased with or without stimulation in lymphocytes from patients
with ABD. Numbers of IFN- γ and IL-2-producing T cells were increased in peripheral
blood from patients with ABD, and were correlated with disease activity [16, 17]. High-
15 level production of IFN- γ was also induced by BD113-20. Bacterial stimulation may
enhance Th-1 cell-mediated immunity in patients with ABD, resulting in the progression
of chronic inflammation. We are now studying mechanisms by which production of
IFN- γ and IL-12 is induced by BD113-20.

IL-10 is secreted from Th-2 cells, and it down-regulates Th-1 immunity and
20 inflammation. IL-10 production in the patients and controls was at a low level and
variable. We recognized no clear tendency in IL-10 production. IL-4 is also produced by
Th-2 cells, however its level was below the detection limit in this study. Serum IL-4, IL-
10, and IL-13 levels in patients with ABD were detectable but the pathogenic roles of
Th-2-associating cytokines are still not clear [18, 19]. Our study indicates that the Th-2

humoral immunity against oral *Streptococcus* may be diverse in individuals, and it may not be effective for a case of stimulation with streptococcal antigens. On the other hand, autoantibodies against heat shock proteins (65kDa and ab-crystallin), which may be induced by bacterial stimulation, and endothelial cells have been detected in patients
5 with ABD [20,21,22]. Although humoral immunity may play an important pathogenic role in ABD, any association with bacterial stimulation and the induction of autoantibodies is still obscure.

Some papers indicated that cytokine profiles in serum and/or production from PBMC are different in disease activity [18, 23]. PBMC from patients with ABD
10 produced higher levels IL-4, IL-10, and IL-13, and the additions of IFN- γ to the anti-CD3 stimulated PBMC changed Th-1/Th-2 pattern with enhanced IL-12 and decreased IL-4 and IL-10 production [19]. Number of cell spontaneously secreting IFN- γ IL-12, and TNF- α were increased in patients with active ABD [23]. In contrast, our result indicated that cytokine pattern was not different in the inactive and active patients.
15 Some researchers in the Japanese Behçet's disease committee reported that PBMCs from patients were already activated *in vivo* by some antigen. They reported that lymphocytes had been produced IL-8 or TNF for 1-2 days without stimuli (personal communication). Other papers also indicated that lymphocytes from patients with ABD were activated by some antigens *in vivo*. To eliminate the influence of original stimuli in
20 the patients, PBMCs were washed three times and cultured for one week in this study. The PBMC from both inactive and active patients was produced high IL-8 and Th-1 associated cytokines. The immunological future against antigen stimulations may be important for host factor of induction of ABD.

In conclusion, *S. sanguinis* (BD113-20) isolated from a patient with ABD had

the capacity to stimulate activated neutrophils. Lymphocytes from patients with ABD showed high levels of production of IL-8, IFN- γ and IL-12 due to the neutrophil-activating bacteria. This study suggests that both bacterial stimulation and host hypersensitivity are involved in the symptoms and pathogenesis of ABD.

5

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Table 1. Historical information of patients with ABD

Patient number	Age	Sex	Age when first symptoms appeared	First symptoms
1	32	m	20	oral ulcer
2	54	m	49	oral ulcer, EN ^a , arthritis
3	49	f	32	oral ulcer, uveitis
4	32	m	30	uveitis
5	43	f	23	uveitis
6	73	f	60	uveitis
7	79	f	68	EN
8	71	m	43	uveitis
9	68	m	43	oral ulcer, papula
10	52	m	32	oral ulcer
11	45	f	34	oral ulcer, EN, genital ulcer
12	45	m	34	oral ulcer, uveitis
13	64	f	37	uveitis
14	62	f	35	uveitis, EN
15	61	m	23	oral ulcer
16	49	f	33	oral ulcer
17	49	f	33	oral ulcer
18	62	f	37	oral ulcer
19	56	f	32	oral ulcer

^aEN; erythema nodosum

Table 2. Clinical data of patients with ABD.

Patient Number	Present symptoms except for uveitis	Treatment	ESR ^b	CRP	ASO	Activity
1	oral ulcer	colchicine	16	+	-	oral ulcer, uveitis
2	oral ulcer, EN ^a , arthritis, genital ulcer	steroid (instillation), colchicine	-	-	NT ^c	EN
3	oral ulcer, EN, arthritis	steroid, cyclosporine, colchicine	-	-	-	-
4	oral ulcer, EN, arthritis, epididymitis	steroid, colchicine	-	-	NT	uveitis
5	oral ulcer, erythema,	steroid, colchicine	-	-	-	oral ulcer, uveitis
6	oral ulcer, arthritis	steroid, colchicine	-	-	NT	oral ulcer, uveitis
7	oral ulcer, EN, arthritis	steroid, colchicine	-	-	NT	oral ulcer, uveitis
8	oral ulcer, genital ulcer	steroid (instillation), colchicine	-	+	NT	oral ulcer, EN
9	oral ulcer, EN, arthritis, genital ulcer	colchicine	-	-	NT	oral ulcer
10	oral ulcer, genital ulcer	(-)	-	-	NT	-
11	oral ulcer, EN, genital ulcer	cyclosporine, colchicine	-	+	NT	uveitis
12	oral ulcer, EN	cyclosporine, colchicine	41	+	NT	uveitis
13	(-)	steroid (active symptoms)	-	+	NT	-
14	EN	steroid (active symptoms)	-	+	NT	-
15	oral ulcer	colchicine	-	-	NT	-
16	oral ulcer	colchicine	16	-	NT	-
17	oral ulcer, EN	steroid, colchicine	-	-	-	-
18	oral ulcer, genital ulcer	steroid (instillation), colchicine	-	-	NT	-
19	oral ulcer, EN	colchicine	28	-	-	EN, uveitis

^aEN; erythema nodosum, ^bESR; erythrocyte sedimentation rate (mm/hr), ^cNT=not tested

Figure legends

Fig. 1. Chemiluminescence assay with various antigens

Whole blood obtained from patients with ABD (n=19) and healthy volunteers (n=21) was stimulated with zymozan and *Streptococcus* antigens. Asterisks (*) indicate significantly (p<0.05) lower values than in both the patients and controls stimulated with antigen of strain BD113-20 (a strain isolated from a patient with ABD).

Fig. 2. IL-8 production by peripheral lymphocytes

Lymphocytes from the patients (ABD; n=19) and controls (n=21) were stimulated with *S. sanguinis* (ATCC10556 or strain BD113-20) or conA. Asterisks (*) with bars indicate significant differences (p<0.05).

Fig. 3. IFN- γ production by peripheral lymphocytes

Lymphocytes from the patients (ABD; n=19) and healthy controls (n=21) were stimulated with *S. sanguinis* (ATCC10556 or strain BD113-20) or conA. Asterisks (*) with bars indicate significant differences (p<0.05).

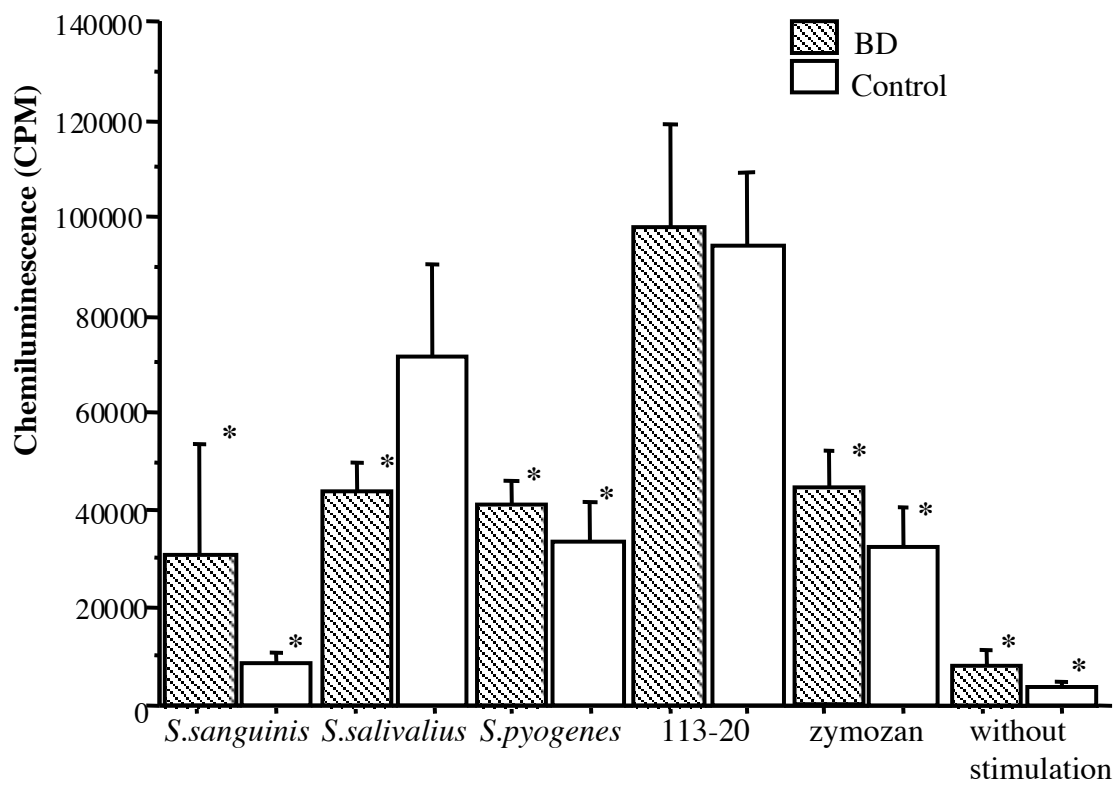
Figure 4. IL-12 production from peripheral lymphocytes

Lymphocytes from the patients (ABD; n=19) and controls (n=21) were stimulated with *S. sanguinis* (ATCC10556 or strain BD113-20) or conA. Asterisks (*) with bars indicate significant differences (p<0.05).

Fig. 5. IL-10 production by peripheral lymphocytes

Lymphocytes from the patients (ABD; n=19) and controls (n=21) were stimulated with *S. sanguinis* (ATCC10556 or strain BD113-20) or conA. Asterisks (*) with bars indicate significant differences (p<0.05).

Fig. 1



5

Fig. 2

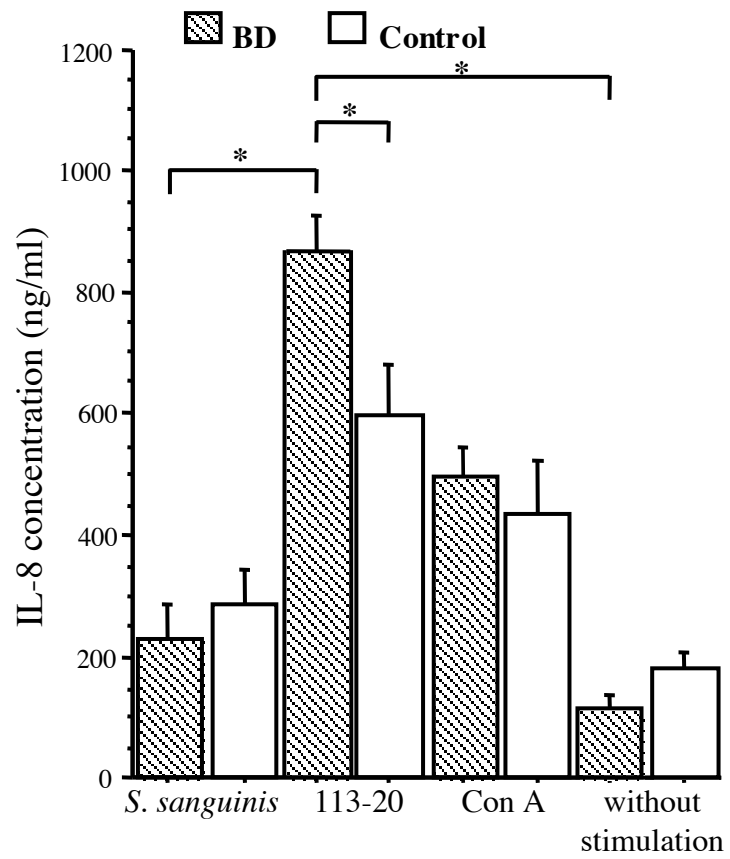


Fig. 3

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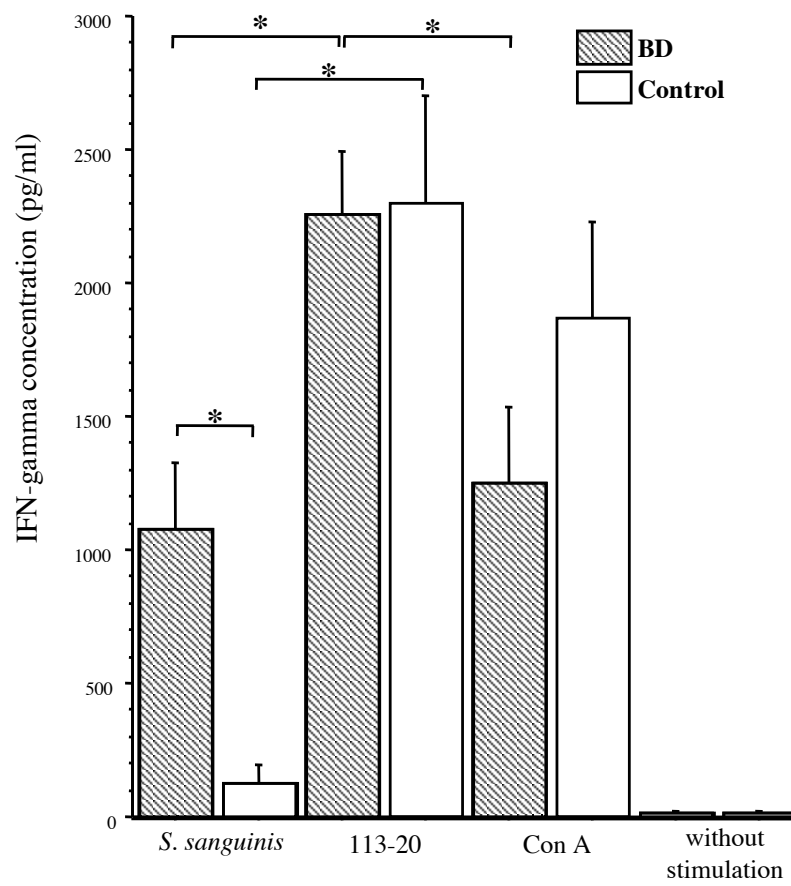
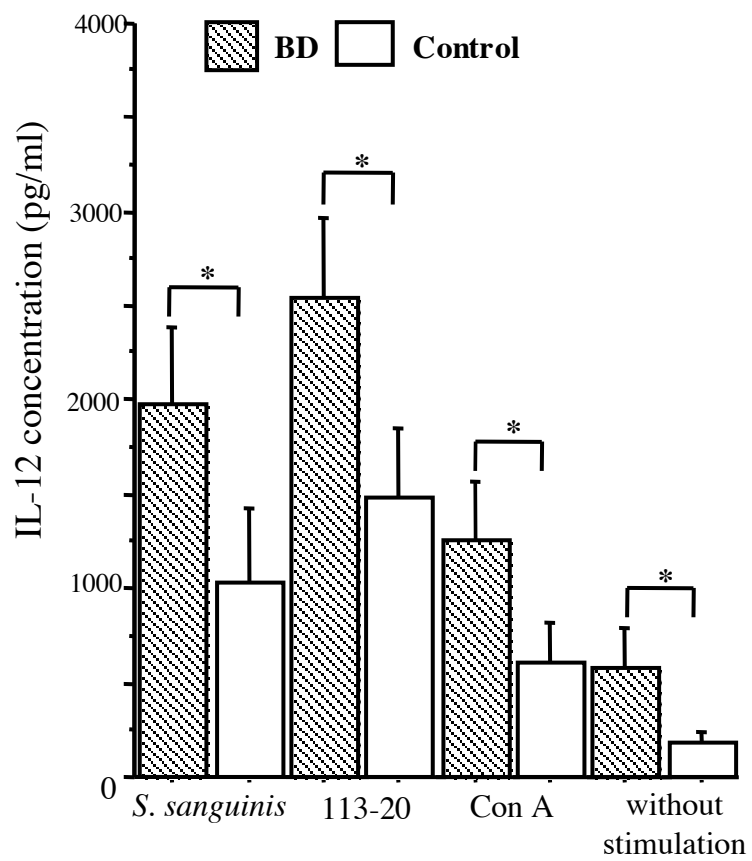


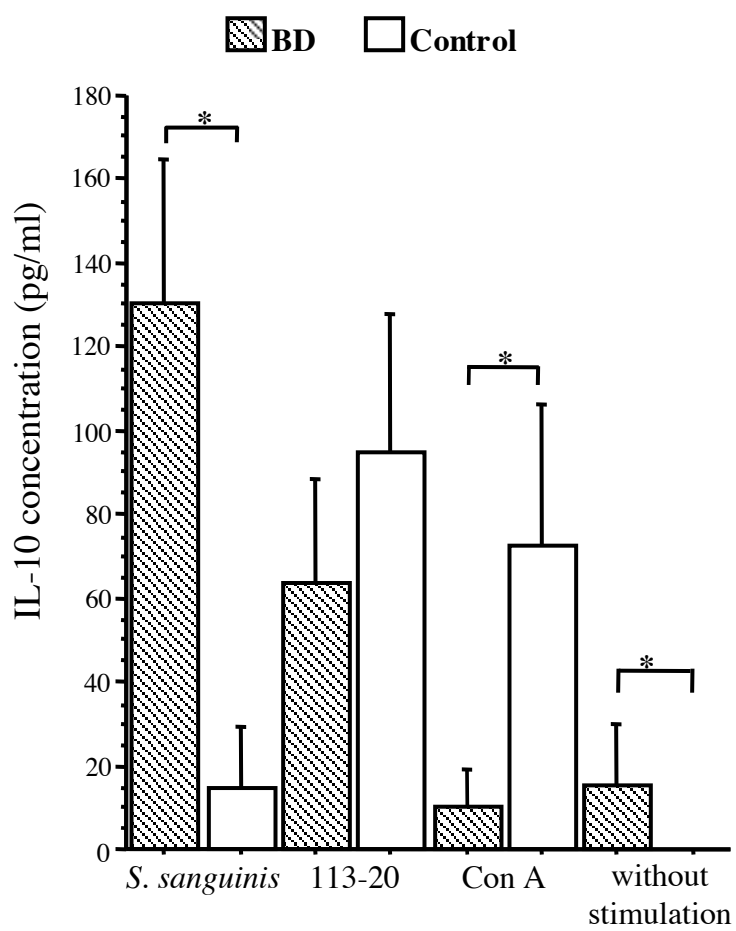
Fig. 4



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Fig. 5

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