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Serum of Behçet's Disease Enhances Superoxide Production of Normal Neutrophils

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Using an MCLA-dependent chemiluminescence technique, we evaluated superoxide production by neutrophils isolated from 7 patients with Behçet's disease. After stimulation by phorbol myristate acetate, N-formyl-methionyl-leucyl-phenylalanine or opsonized zymosan, neutrophils from the patients produced significantly more superoxide than those from 20 controls. Pretreatment of control neutrophils with serum prominently enhanced superoxide production, and serum from Behçet's disease patients had a significantly greater effect than that from controls. These findings suggest that serum from patients with Behçet disease contains the priming factor(s) that can enhance enhanced superoxide production by neutrophils in response to stimulation.

Keywords: Behçet's disease, superoxide, neutrophils, priming factor(s), MCLA-dependent chemiluminescence technique

INTRODUCTION

Behçet's disease (BD) was first described by Behçet in 1937, as a disorder featuring the clinical triad of oral ulceration, genital ulceration, and hypopyon-iritis.[1] Subsequently, the clinical features of this disease have been well established (chorioretinitis, erythema nodosum, thrombophlebitis, arthritis, orchiepididymitis, and central nervous system, gastrointestinal, and pulmonary involvements).[2] Although the etiopathogenesis of this disease has not yet been clarified, viral, environmental, or genetic factors, and immune dysregulation have been suggested to have a role. Histopathological examination shows cellular infiltration in the majority of Behçet's lesions, consisting of lymphocytes, plasma cells, monocytes, and neutrophils in varying proportions, depending on the stage of the lesions.

Since the discovery by Babior *et al.*[3] that phagocytozing neutrophils produce substantial quantities of superoxide radicals, numerous studies have focused on potential toxicity of these radicals in inflamed tissues[4–6] and on superoxide dismutase, an important superoxide scavenging enzyme.[5,7] It has been reported that various

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functions of peripheral blood neutrophils, such as chemotaxis, phagocytosis, and superoxide generation, are increased in BD.[8–11] This potentiation of neutrophil function has been suggested to play a causative role in BD.[9] In the present study, we investigated whether superoxide release from neutrophils is actually enhanced in BD. Since some cytokines are known to potentiate neutrophil function,[12,13] we also examined the priming effect of serum from BD patients on superoxide production in response to several stimuli.

MATERIALS AND METHODS

Patients

Seven Japanese patients with BD (5 men and 2 women, mean age : 46.3 years; range : 29 to 64 years) were studied. The criteria for diagnosing the disease were those proposed by the International Study Group for BD.[2] Their clinical details and medications at the time of examining the blood sample are shown in Table I. Twenty other subjects were enrolled as controls (14 men and 6 women, mean age : 43.6 years; range : 26 to 69 years), including 12 disease controls with amyotrophic lateral sclerosis (n = 6), epilepsy (n = 1), noninflammatory myopathy (n = 2), or spinocerebellar degeneration (n = 3), and 8 healthy volunteers. The controls were not taking steroids or colchicine.

Materials

Phorbol myristate acetate (PMA), N-formyl-methionyl-leucyl-phenylalanine (FMLP), and zymosan A were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). PMA and FMLP were dissolved in 50% DMSO and 50% Hanks' balanced salt solution without phenol red (HBSS, pH 7.4), and were stored at –80°C.[14,15] Opsonized zymosan (OZ) was prepared from zymosan A using pooled fresh human serum, and was stored at –80°C for no longer than three weeks until use.[14,15] 2-Methyl-6-(p methoxyphenyl)-3,7-dihydroimidazo[1,2-a]pyrazin-3-one (MCLA) was purchased from Tokyo Kasei (Japan) and prepared by the method described previously.[14,15]

Cell Isolation

Leukocytes were isolated from the peripheral blood of patients and controls.[14] The leukocytes thus obtained contained 65–91% neutrophils, and were suspended in HBSS (pH 7.4) and kept at 4°C for no longer than 3 h prior to use.

Measurement of Superoxide Production

Superoxide production was determined by the MCLA-dependent chemiluminescence method using a BLR 301 Luminescence Reader (Aloka, Japan). All experiments were carried out in the

TABLE I Details of patients with BD

No.	Sex	Age	Lesions					Medication
			oral	genital	eye	skin	CNS	
1	M	29	(+)	(+)	(+)	(+)	+	steroid immunosuppressant
2	M	41	+	–	+	+	+	steroid colchicine
3	M	35	(+)	(+)	(+)	–	(+)	–
4	F	63	+	(+)	(+)	(+)	(+)	aspirin
5	F	48	+	(+)	–	+	(+)	steroid aspirin
6	M	42	+	–	–	+	–	aspirin colchicine
7	M	64	+	–	+	+	–	steroid colchicine

(+): The symptom was not active at the time of examining the blood sample.
CNS: central nervous system.

incubation chamber of the Luminescence Reader at 37°C in a total volume of 2 ml. The reaction mixture for the basal system consisted of neutrophils (5.0×10^4 cells/ml) and 1.0 μM MCLA in HBSS (pH 7.4) with preincubation for 5 min at 37°C. The reaction was started by addition of each of the stimulants (0.1 μg/ml PMA, 0.5 mg/ml OZ, or 1.0 μM FMLP). Superoxide production was expressed as photon counts per min. The maximum photon count for each reaction and the integrated photon count for the whole incubation period were defined as the peak height (PH) and the integrated photon count (IPC), respectively. The incubation period was 10 min for PMA or OZ and 5 min for FMLP.

Pretreatment of Neutrophils with Serum

Neutrophils (1.0×10^6 cells) isolated from a control were incubated with various concentrations of serum obtained from each of the patients and controls for 30 min at 37°C in HBSS (pH 7.4). Then the pretreated mixture was rapidly cooled to 4°C and centrifuged for 5 min at 250 × g at 4°C. Next, the neutrophils were washed twice at 4°C in HBSS without calcium and magnesium, and then suspended in HBSS. The superoxide production of the pretreated neutrophils was determined by the chemiluminescence method described above. When the neutrophils of a control were pretreated, the serum used was obtained from other controls.

RESULTS

BD neutrophils without serum pretreatment showed a high level of superoxide release in response to PMA, OZ, or FMLP. The PH and IPC for BD neutrophils were significantly higher than those for control neutrophils (Table II). Preincubation with serum increased superoxide production by control neutrophils. The maximum PH and IPC were reached when the serum concentration was 20% or more (Figure 1). Therefore, the serum concentration for pretreatment was fixed at 50% in subsequent experiments. The PMA-stimulated PH and IPC or FMLP-stimulated PH and IPC of control neutrophils pretreated by patient serum were significantly increased compared with those of control neutrophils pretreated by control serum (Table III). The PH and IPC of neutrophils from one control showed no significant difference when incubation was done with serum from each of the other controls.

DISCUSSION

Enhanced superoxide production by neutrophils in BD was reported previously.[9–11] In the present study, we confirmed increased superoxide production by PMA-, OZ-, and FMLP-stimulated neutrophils using the MCLA-dependent chemiluminescence method. Some previous reports have suggested that superoxide production by neutrophils is higher during exacerbations than

TABLE II Superoxide production by neutrophils without pretreatment

Neutrophils	PMA		OZ		FMLP	
	PH	IPC	PH	IPC	PH	IPC
control	960	5,209	728	3,882	345	383
(n = 20)	± 412	± 213	± 265	± 2,105	± 146	± 194
BD	1,610	8,558	1,255	6,796	695	1,042
(n = 7)	± 479	± 2,184	± 374	± 2,585	± 291	± 475
p value	<0.01	<0.01	<0.01	<0.05	<0.01	<0.01

Results in kcpm were expressed as mean ± SD.
The p values were assessed by Mann-Whitney U test.

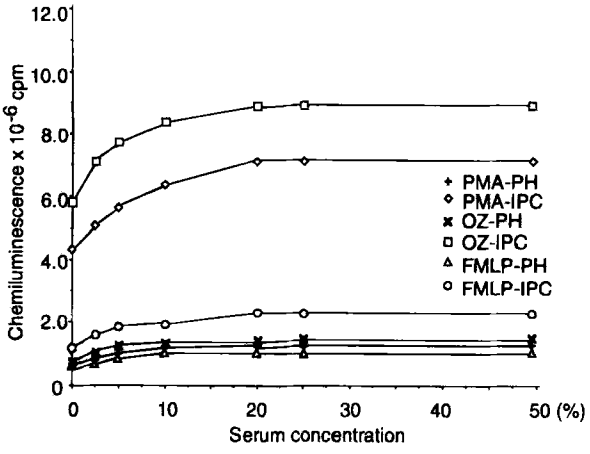

FIGURE 1 Effect of serum concentration on superoxide production of neutrophils. Representative data from control's neutrophils pretreated with another control's serum at various concentrations are illustrated. Superoxide production was increased with increasing serum concentrations up to 20%, and at higher concentrations it was not increased any more. This serum concentration-related pattern of superoxide production was observed similarly in all subjects. PMA-PH and PMA-IPC indicate the peak height (PH) and the integrated photon count (IPC), respectively, obtained from PMA-stimulated neutrophils. OZ-PH, OZ-IPC, FMLP-PH and FMLP-IPC are also defined in a similar way.

during remissions of BD.[11] We did not compare active BD with inactive BD in the present study, since the number of patients was too small and since patients with active BD tend to be treated with steroids or colchicine which may have an inhibitory effect on neutrophil superoxide production.[11,16] Moreover, we found it difficult to draw a distinction between active and inactive BD. However, one patient (No. 1 in Table I) in our series suggested a possible relationship between disease activity and neutrophil superoxide production. He showed the most severe central nervous system (CNS) symptoms and the highest neutrophil superoxide production among all the patients we examined. At the time his neutrophils were tested, however, he did not have all four major signs of active BD.[2] According to Pronai *et al.*[11] and Niwa *et al.*,[9] his BD should have been classified as inactive despite severe CNS symptoms.

The MCLA-dependent chemiluminescence assay used in the present study is a very reliable method for quantitating superoxide production by neutrophils. However, MCLA is a chemiluminescence probe which can respond to singlet oxygen as well as superoxide.[17] Singlet oxygen is released by eosinophils.[18] Under our experimental conditions, the population of these cells was very small and the chemiluminescence produced by the various stimulants was completely abolished by the addition of superoxide dismutase.[19] Therefore, the contribution of singlet oxygen to the chemiluminescence detected in our study was thought to be negligible.

The most important finding in the present study is the priming effect of BD serum. Some previous reports have suggested that enhanced superoxide production by BD neutrophils may be due to primary dysfunction of the neutrophils or may be secondary to bioactive substances in the serum. The present study showed that control neutrophils preincubated with BD serum released sig-

TABLE III Superoxide production by pretreated neutrophils

Serum	PMA		OZ		FMLP	
	PH	IPC	PH	IPC	PH	IPC
control	1,466	6,929	1,512	8,902	1,350	2,079
(n = 7)	± 641	± 3,064	± 676	± 3,657	± 862	± 1,494
BD	1,684	7,738	1,479	8,996	1,650	2,507
(n = 7)	± 744	± 3,023	± 512	± 2,936	± 771	± 1,279
p value	<0.05	<0.05	NS	NS	<0.05	<0.05

Results in kcpm were expressed as mean ± SD.
The p values were assessed by Wilcoxon signed-rank test.
NS: no significance.

nificantly more superoxide in response to some of the stimulants than neutrophils preincubated with control serum, indicating that priming factor(s) may be present in the serum of BD patients. PMA-stimulated PH and IPC as well as FMLP-stimulated PH and IPC were significantly increased by pretreatment with patient serum, while OZ-stimulated PH and IPC were not. This difference may be derived from the mechanisms by which the stimulants act. PMA acts directly on protein kinase C, and FMLP induces signal transduction from its cell membrane receptor to protein kinase C. OZ is prepared by incubation with pooled fresh serum (i.e., opsonization) and is recognized by phagocytes and then incorporated by these cells. This immunological recognition on the cell surface seems to trigger the superoxide production. The exposure of neutrophils to serum may have a similar effect. Immunoglobulins and related serum factors may modulate neutrophil surface receptors, and may thus lessen the difference in the response to OZ.

There have been many studies on abnormal chemotaxis by BD polymorphonuclear leukocytes (PMN). Some authors have attributed the enhanced chemotaxis to a primary functional abnormality of PMN,[8,20–22] while others have blamed priming factors in patient serum.[8] The superoxide scavenging activity (SSA)[23] and the activities of superoxide dismutase, catalase, myeloperoxidase and glutathione peroxidase[24] in neutrophils are significantly decreased in BD patients as compared with healthy controls. These findings suggest that BD neutrophils are vulnerable to oxidative injury, but are not likely to have a direct influence on increased superoxide release. The plasma level of SSA is reported to be lower in BD patients than in controls,[23] although the reason is not clear. Incubation with plasma having a low SSA may possibly induce some functional changes in neutrophils. In our preliminary experiments, the enhancement of superoxide production did not show a significant difference between pretreatment with serum or plasma from BD patients (data not shown).

The present study did not define the priming factor(s), but the differences between patient and control serum may provide some clues. The plasma fibrinogen level is significantly higher in BD patients than in controls.[24–26] However, our experiments excluded fibrinogen and related constituents of the fibrin net by using serum instead of plasma. Circulating immune complexes, which are found in 46% of BD patients,[27] may be a priming factor. However, an antibody-related priming effect and neutrophil activation have only been reported for anti-neutrophil cytoplasmic autoantibodies (ANCA)[28] and there is no evidence that ANCA are present in BD. Moreover, γ-interferon (produced by T lymphocytes) is significantly increased in BD serum.[29,30] When cultured T lymphocytes from BD patients were stimulated by T cell mitogens or by streptococcal preparations, the supernatants from these cultures showed significantly increased interleukin-1 like activity. However, the changes of neutrophil in chemotaxis, phagocytosis, and superoxide generation induced by incubation with the supernatant of cultured T lymphocytes were not affected by antisera against human interleukin(IL)-1, IL-2, γ-interferon, or tumor necrosis factor (TNF).[12] BD neutrophils stimulated by lipopolysaccharide produced significantly more IL-1, IL-6, IL-8, and TNF than control neutrophils, although the cytokine levels showed no correlation with BD activity.[13] Although we could not identify the priming factor(s), the candidate(s) may be found in some products of activated lymphocytes or macrophages.

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