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Adamantiades-Behçet's disease: interleukin-8 is increased in serum of patients with active oral and neurological manifestations and is secreted by small vessel endothelial cells

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Abstract **The serum levels of several cytokines were determined in 94 patients with Adamantiades-Behçet's disease (ABD), aged 36.1 ± 11.0 years, during the active stage (n = 75) and the inactive stage (n = 19) of the disease. A group of 75 healthy individuals matched for age and sex served as controls. Cytokine levels were determined using commercially available ELISA kits. Of the 75 patients with active disease and 19 with inactive disease, 38 (51%) and 4 (21%), respectively, and 23 healthy controls (31%) were found to have detectable levels of interleukin 8 (IL-8) in their serum ($P < 0.05$). Also, increased IL-8 serum levels were found in patients with active disease (median 12 pg/ml, $P = 0.010$) compared to patients with inactive disease (≤ 10 pg/ml) and to healthy controls (≤ 10 pg/ml). In particular, patients with oral aphthous ulcers (n = 51, 34 pg/ml) and neurological features (n = 4, 71 pg/ml) exhibited increased IL-8 levels. In contrast, there was no correlation between disease activity and the serum levels of IL-1α, IL-1β, tumor necrosis factor alpha (TNF-α), soluble intercellular adhesion molecule-1 or basic fibroblast growth factor (bFGF). In a second set of experiments, the involvement of dermal microvascular endothelial cells in IL-8 secretion was investigated. Immortalized human dermal microvascular endothelial cells (HMEC-1 cells) were maintained for 4 h in vitro with serum from 18 ABD patients or with IL-1β, a known stimulator of IL-8 synthesis, TNF-α or their combination at five- to tenfold higher concentrations than those found in the serum of ABD patients. Increased IL-8 secretion was found after incubation with ABD patients' serum (median 20 pg/ml), but IL-1β, TNF-α and IL-1β + TNF-α failed to induce IL-8 secretion by HMEC-1 cells (≤ 1–1.2 pg/ml) in biologically relevant concentrations. Our study showed increased IL-8 serum levels in ABD patients with active oral and neurological manifestations. Human microvascular endothelial cells may, at least partially, be responsible for the enhanced IL-8 secretion in the active stage of the disease.**

Key words Behçet's disease · Interleukin-8 · Endothelial cells

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Introduction

Adamantiades-Behçet's disease (Behçet's disease, ABD) is a multisystemic, relapsing vasculitis of unknown etiology with a chronic course and, occasionally, a severe prognosis. The disorder was first recognized by B. Adamantiades in 1931 [1] and described extensively by H. Behçet in 1937 [2]. Diagnosis is based on clinical criteria, since specific laboratory parameters are still lacking [3, 4].

The evidence is strong that immunological mechanisms are involved in the pathogenesis of the disease [5]. Recent studies have been focused on the correlation of various cytokines with ABD and its severity [6–10]. Since alterations in small vessel walls are often the initial pathological sign [11–13], the investigation of biological interactions between vascular endothelial cells and cytokines is likely to increase our understanding of the inflammatory vascular process that takes place in this peculiar disorder.

Interleukin-8 (IL-8), a major chemokine known to attract and activate leukocytes [14, 15], has been assumed to represent such a notable link between immune system activation and endothelial alterations in ABD. Increased IL-8 levels have been found in ABD patients' serum [16–

19] produced by peripheral monocytes [9], neutrophils [20] and T cells in skin lesions [21]. In addition, immunohistochemical studies in skin lesions of ABD patients have indicated IL-8 synthesis not only in mononuclear cells but also in fibroblasts and, more interestingly, in dermal microvascular endothelial cells [20].

Based on this background, the aim of the first group of experiments in the present study was to evaluate the correlation between IL-8 serum levels in ABD patients and the activity of the disease and its different clinical manifestations. In addition, the involvement of cytokines, such as IL-1α, IL-1β, tumor necrosis factor alfa (TNF-α), soluble intercellular adhesion molecule-1 (sICAM-1) and basic fibroblast growth factor (bFGF), which have been reported to be involved in the development of several inflammatory disorders, in ABD was investigated. In a second set of experiments, we sought to determine whether human dermal microvascular endothelial cells can be triggered by patients' serum to secrete IL-8.

Patients and methods

Serum cytokine levels in ABD patients

Cytokine levels were determined in serum samples from 94 untreated ABD patients (56 male, 38 female; aged 36.1 ± 11.0 years) of German, Greek and Turkish origin. Diagnosis was based on the criteria of the International Study Group for Behçet's Disease [3]. Serum samples were drawn during clinically active (one or more clinical manifestations present) (75 patients; 43 male, 32 female; aged 35.3 ± 10.8 years) and inactive disease (no clinical sign present) (19 patients; 13 male, 6 female; aged 39.5 ± 11.4 years). The clinical manifestations of the patients with active ABD are presented in Table 1. Serum samples from 75 healthy individuals matched for sex and age (44 male, 31 female; aged 36.0 ± 11.5 years) served as the control group. Venous blood samples used were drawn within similar time frames, centrifuged at 2500 rpm for 10 min to separate the serum which was aliquoted and stored at –70 °C until use.

IL-8 secretion by skin vessel endothelial cells in vitro

Immortalized human dermal microvascular endothelial cells (HMEC-1 cells; a gift from Dr. Ades, Emory University, Atlanta, Ga.), shown to exhibit characteristics and functions similar to those of human dermal microvascular endothelial cells [22], were maintained in endothelial basal medium (Clonetics, San Diego, Calif.) supplemented with 2% fetal calf serum, 10 ng/ml epidermal growth factor, 1 μg/ml hydrocortisone, 50 μg/ml gentamicin, 50 ng/ml amphotericin and 2 ml bovine brain extract (all Clonetics) at 37 °C in an atmosphere containing 5% CO_2. Before proceeding to the in vitro experiments, confluent HMEC-1 cells in six-well plates (Becton Dickinson, Plymouth, UK) were adapted for 2 days to endothelial basal medium without additives and washed twice with phosphate-buffered saline (PBS) without Ca^{2+} or Mg^{2+} (Biochrom, Berlin, Germany).

The serum samples from 18 patients (13 male, 5 female; aged 34.6 ± 9.4 years), representing serum cytokine variations detected in the first set of experiments, were selected for in vitro investigations. The serum from each ABD patient was diluted 1:1 with Dulbecco's modified Eagle's medium (DMEM) and incubated with HMEC-1 cells for 4 h, which was found in preliminary experiments to be the optimal incubation time for IL-8 secretion by HMEC-1 cells. In parallel experiments, HMEC-1 cells were incubated in DMEM supplemented with 10 pg/ml IL-1β, 5 pg/ml TNF-α (both from R & D Systems, Wiesbaden, Germany) or with the combination of IL-1β (10 pg/ml) and TNF-α (5 pg/ml) for 4 h.

In additional experiments, HMEC-1 cells were incubated with IL-1β (0.1, 1 or 10 ng/ml), TNF-α (0.5, 5 or 50 ng/ml), IL-8 (1, 10 or 100 ng/ml) or bFGF (1, 10, or 100 ng/ml) (all from R & D Systems) for 4 h. HMEC-1 cells incubated with DMEM without additives served as controls.

At the end of incubation with serum or cytokines, the medium was aspirated, the HMEC-1 cells were washed twice with PBS and incubated in fresh DMEM without additives for 24 h. The latter supernatants were harvested and aliquots were drawn for evaluation. Cell viability was assessed by trypan blue staining and was found to be > 95% in all experiments.

Evaluation of cytokine levels

Levels of IL-8, IL-1α, IL-1β, TNF-α, sICAM-1 and bFGF in serum samples were measured using commercially available enzyme-linked immunosorbent assays (Quantikine ELISA-kits, R & D Systems). Levels of IL-8 in medium samples were measured using a modified ELISA to increase sensitivity. Optical density was measured at 405 nm in a Dynatech ELISA reader.

Statistics

Data are presented as median values and their 25–75% ranges, unless stated otherwise. Some of the in vitro data are presented as means ± standard deviation, being representative of three distinct experiments, except where otherwise stated. Statistical significance was evaluated by computer-assisted analysis using the chi-squared test, Mann-Whitney-Wilcoxon *U*-test, linear regression and plot evaluation of data or the two-sided Student's *t*-test.

Table 1 Clinical features of patients with active ABD

Clinical manifestation	Patients (n = 75)	
	Number	%
Mucocutaneous signs	65	87
Oral aphthous ulcers	51	68
Genital ulcers	14	19
Erythema nodosum	11	15
Sterile pustules/papules	20	27
Ocular lesions	26	35
Arthritis/arthropathy	22	29
Vascular lesions	2	3
Central nervous system involvement	4	5
Other	3	4

Results

Cytokine levels in serum from ABD patients

Of the 75 patients with active ABD, 38 (51%) had detectable levels of IL-8 in their serum (detection limit 10 ng/ml), while only 4 of 19 patients in the inactive phase (21%) and 23 of 75 healthy controls (31%) exhibited detectable levels ($P < 0.05$). In addition, serum levels of IL-8 were found to be significantly elevated in patients with active ABD (median value 12 pg/ml, 25–75% range ≤ 10–56 pg/ml; $P = 0.010$) in comparison to those with inactive ABD (≤ 10 pg/ml, 25–75% range ≤ 10 pg/ml) and to healthy controls (≤ 10 pg/ml, ≤ 10–33 pg/ml; Table 2). Concern-

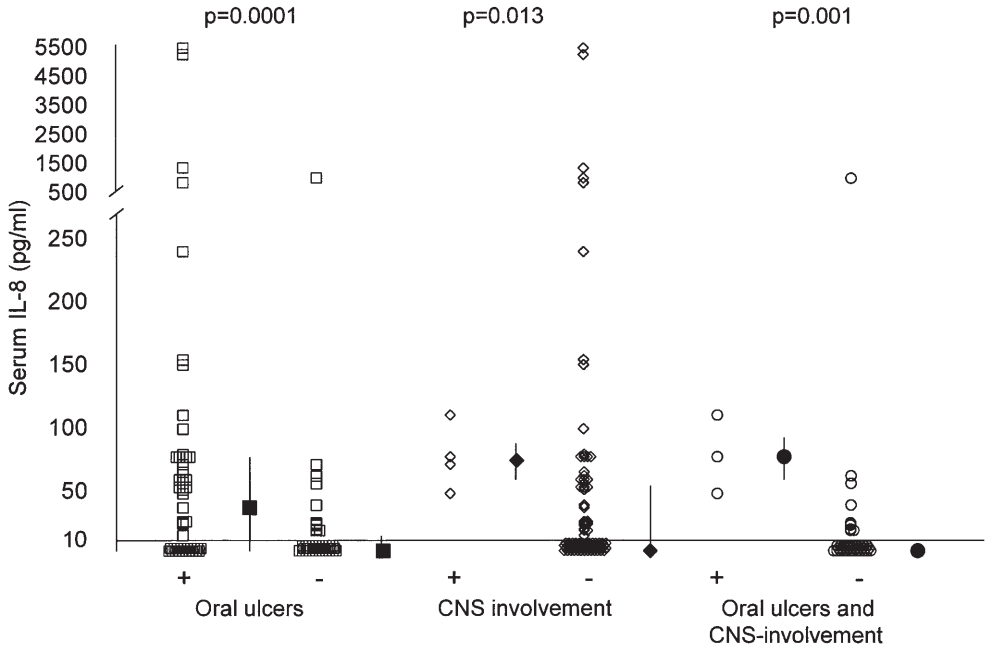

Fig. 1 IL-8 levels in serum from patients with or without active oral aphthous ulcers, with or without active involvement of the central nervous system, and with active oral aphthous ulcers and involvement of the central nervous system as well as without either clinical manifestation. IL-8 levels are presented as absolute values, median values of the groups and their 25% and 75% ranges

Table 2 Cytokine levels in serum from ABD patients. Data are presented as median values and their 25% and 75% ranges (*ND* not done, *NS* not significant)

Cytokine	Patients with active disease		Patients with inactive disease		Healthy controls		Significance
	Median	Quartiles	Median	Quartiles	Median	Quartiles	
IL-8 (pg/ml)	12	≤ 10 –56	≤ 10	≤ 10	≤ 10	≤ 10 –33	$P = 0.010$
IL-1α (pg/ml)	0.8	0.7– 0.9	0.8	0.8–0.9	ND		NS
IL-1β (pg/ml)	0.4	0.1– 0.7	0.3	0.1–0.5	0.5	0.3– 1.0	$P = 0.039$
TNF-α (pg/ml)	1.1	0.8– 1.8	ND		1.1	0.5– 2.0	NS
bFGF (pg/ml)	2.7	1.0– 8.9	4.2	1.3–6.0	2.8	0.9– 8.3	NS
sICAM-1 (ng/ml)	10	4.3–14	ND		12	9.5–13	NS

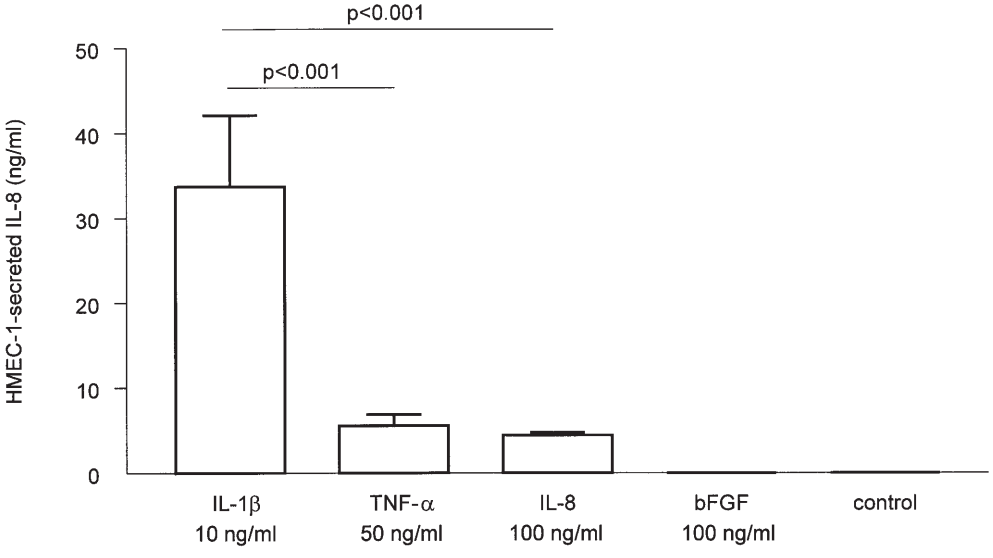

Fig. 2 IL-8 secretion by HMEC-1 cells induced by high levels of IL-1β (10 ng/ml), TNF-α (50 ng/ml), IL-8 (100 ng/ml) and bFGF (100 ng/ml) and in control cultures. IL-8 levels are presented as means ± SD

ing the association of serum IL-8 levels with the profile of active clinical manifestations, patients with oral aphthous ulcers ($n = 51$; 34 pg/ml, ≤ 10–74 pg/ml), involvement of the central nervous system ($n = 4$; 71 pg/ml; 62–82 pg/ml) or both manifestations ($n = 3$; 74 pg/ml, 60–91 pg/ml) exhibited characteristically increased serum IL-8 levels compared to those without oral aphthous ulcers ($n = 43$; ≤ 10 pg/ml, ≤ 10–16 pg/ml; $P = 0.0001$), without neurolog-

ical signs (n = 90; ≤ 10 pg/ml, ≤ 10–50 pg/ml; P = 0.013) or without both manifestations (n = 42; ≤ 10 pg/ml, 25% and 75% range ≤ 10 pg/ml; P = 0.001), respectively (Fig. 1). No correlation was found between the IL-8 serum levels and other clinical manifestations.

In contrast, there was no correlation between serum levels of IL-1α, TNF-α, sICAM-1 or bFGF and disease activity (Table 2). IL-1β was found to be decreased in patients with ABD compared to the healthy controls (p = 0.039), but there was no difference between patients with active disease and those with inactive disease.

IL-8 secretion by HMEC-1 cells in vitro

HMEC-1 cells were triggered to secrete IL-8 by serum from ABD patients (median value 20 pg/ml, 25–75% range 5.7–55 pg/ml, detection limit 1 pg/ml). In contrast, IL-1β and TNF-α, at concentrations five- to tenfold higher than the serum levels of ABD patients, or their combination, did not increase IL-8 secretion by HMEC-1 cells (≤ 1–1.2 pg/ml). The secretion of IL-8 by HMEC-1 cells after a 4-h incubation with high concentrations of IL-1β (10 ng/ml), TNF-α (50 ng/ml), IL-8 (100 ng/ml) or bFGF (100 ng/ml), being at least 10^4-fold higher than the levels detected in patients' serum is shown in Fig. 2. A strong induction of IL-8 secretion was found after incubation with IL-1β. TNF-α and IL-8 only slightly enhanced IL-8 secretion, while no IL-8 secretion after incubation with bFGF was detected.

Discussion

Chemokines are central to inflammation [23] and there are indications that they play a crucial role in ABD [7, 24]. We present here additional evidence for the involvement of IL-8 in the pathogenesis of the disease and we suggest that endothelial cells could at least partially be responsible for the enhanced IL-8 secretion in the active stage of the disease.

Other cytokines, such as IL-1α, IL-1β, TNF-α, sICAM-1 and bFGF were not increased in patients' serum. IL-1 was investigated because of its capacity to activate lymphocytes and to induce production of other cytokines, such as TNF-α [25] and IL-8 [26]. However, we were unable to confirm previous data indicating increased levels of IL-1β in serum from ABD patients [27]. Special attention was paid to TNF-α, because in a previous study we had observed an increased TNF-α expression in lesional skin biopsies of ABD patients compared with healthy skin of ABD patients, with lesional and healthy skin of patients with leukocytoclastic vasculitis and with skin of healthy controls [28]. However, serum levels of TNF-α did not correlate with enhanced tissue deposition, as previously described [7, 29, 30].

The expression of sICAM-1 on the cell surface is induced by proinflammatory cytokines, such as IL-1 and TNF-α [31]. ICAM-1 is involved in leukocyte extravasation and therefore the release of cell surface ICAM-1, resulting in sICAM-1, may provide a means of controlling adhesive interactions between inflammatory and endothelial cells. Despite the increased levels of sICAM-1 shown in several autoimmune, viral and malignant diseases [32] and in a trial with ABD patients [33], normal sICAM-1 serum levels were found in ABD patients in our studies.

The heparin-binding growth factor, bFGF, is known to stimulate growth of endothelial cells as well as of other cell types, to modulate cell differentiation, to control proliferation and migration of vascular endothelial cells and to play a role in wound healing and tissue repair [34, 35]. Alone or in combination with IL-1 and TNF-α, bFGF can act as a regulator of nerve growth factor production [36]. Although we have found increased serum levels of nerve growth factor in patients with ABD [6], bFGF levels were not elevated.

IL-8 is actively involved in the enhanced adherence of peripheral blood leukocytes to endothelial cells in inflammatory processes [18, 37]. It can be induced by various stimulants, including cytokines, such as IL-1 and TNF-α, as well as viral RNA and DNA fragments [23]. We have previously shown that vasculitic lesions of ABD are characterized by the marked presence of neutrophils and mononuclear cells [38]. Furthermore, we and others have detected an enhanced interaction of patients' lymphocytes with human dermal microvascular endothelial cells in vitro [39, 40], while Sahin et al. [18] have reported enhanced interaction of neutrophils with cultured endothelial cells after pretreatment with serum of patients with ABD. IL-8 secretion after incubation of HMEC-1 cells with serum of ABD patients indicates that chemotaxis is an initial process of inflammation and suggests that serum factor(s), e.g. circulating antiendothelial antibodies [40], may provoke a rapid tissue response in ABD [18, 20]. Such antibodies have been shown to be directed against a disease-specific antigen on human dermal microvascular endothelial cells [41]. These antibodies do not exhibit a cytotoxic effect but activate endothelial cells to produce cytokines.

Since lymphocytes and neutrophils express IL-8 receptors [42, 43], endothelial cell-secreted IL-8 may be regarded as a prominent candidate for the regulation of the inflammatory infiltrates in ABD lesions. ABD seems to be an immune-mediated vasculitis with various tissue targets and the involvement and interrelationship of cytokines could depend on microenvironmental factors associated with each tissue (skin, mucosae, CNS etc.). This may provide a logical connection for the significant correlation between IL-8 levels and oral aphthous ulcers as well as CNS involvement that we observed. However, our own unpublished data do not support a specific correlation between IL-8 and ABD. Patients with active psoriasis also show detectable levels of IL-8 in their serum and their serum induces IL-8 secretion by HMEC-1 cells (data not shown).

The abundance of the various cytokines and chemokines possibly involved in ABD [7] suggests a network interaction as a cause for the immune dysfunction in ABD. Interestingly, IL-1β and TNF-α were not increased in patients' serum and did not induce IL-8 secretion by HMEC-1 as single agents or in combination in biologically relevant concentrations. In addition, neutralizing antibodies against

IL-1β, TNF-α and their combination did not influence IL-8 secretion by HMEC-1 cells induced by serum from one patient with oral aphthous ulcers (data not shown). Only IL-1β was able to induce IL-8 secretion by HMEC-1 cells in markedly high concentrations. It is likely, therefore, that IL-8 secretion in ABD is not mediated by the most prominent proinflammatory cytokines IL-1β and TNF-α or their combination. Another possible candidate, bFGF, failed to induce IL-8 secretion by HMEC-1 cells. Since no other cytokines are known to induce IL-8 secretion by human dermal microvascular endothelial cells, it is possible that the circulating antiendothelial cell antibodies described by Lee et al. [40, 41] may be responsible for this effect.

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