Neutrophil activation in Behcet's disease

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Abstract

Objective

Neutrophils are implicated in the pathogenesis of Behçet's disease (BD). Various functions of neutrophils are studied to clarify this role.

Methods

The oxidative burst and phagocytic functions of neutrophils and surface molecules associated with neutrophil activation (CD10, CD14 and CD16) were investigated in BD patients by flow cytometric methods. Patients with inflammatory arthropathies, sepsis and healthy controls were also studied.

Results

In the oxidative burst experiments, after fMLP and PMA stimulation, stimulation index was found to be significantly decreased in patients both with BD and sepsis compared to healthy controls and inflammatory arthropathies ($p < 0.001$ and $p < 0.01$, respectively). The phagocytosis of labelled E.coli *particles in patients with BD was not different from that of the healthy controls, while it was decreased in diseased controls ($p < 0.001$). The surface density of neutral endopeptidase (CD10) and the mean percentage of LPS receptor (CD14) was found to be significantly higher in both BD patients and diseased controls ($p < 0.001$). The mean percentage of CD16 expression was only low in patients with sepsis ($p < 0.001$), whereas CD16 intensity on cells was found to be lower in patients with BD as well as in sepsis ($p < 0.01$).*

Conclusion

These findings indicate the presence of in vivo *pre-activated neutrophils in BD. A similar activation was also a feature of severe inflammatory disorders.*

Key words

Behçet's disease, neutrophils, CD10, CD14, CD16, phagocytosis, oxidative burst.

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Introduction

Behçet's disease (BD) is a chronic, inflammatory vasculitis characterized by oral and genital ulcerations, ocular and skin lesions, arthritis and neurological involvement (1). The etiology and pathogenesis of BD have not been clarified, but various immunological abnormalities associated with both humoral and cellular immune systems have been defined (2-5).

Neutrophils play a pivotal role in innate immune response. They are the most abundant leucocytes with their short lifespan and serve as a first line of defense against infectious agents. Neutrophils respond rapidly to chemotactic stimuli, phagocyte and destroy foreign particles using their oxidative and non-oxidative destroying mechanisms and then eliminated. Functional abnormalities in one of these steps may cause subsequent defective response in immune system.

Functional status of neutrophils can be evaluated by various methods. Flow cytometric approach is a fast and simple method to evaluate some of neutrophil functions such as phagocytosis and oxidative burst (6). This method also enables the evaluation of neutrophil surface antigens at the same time.

Cell surface antigens expressed on neutrophils such as CD10, CD14 and CD16 are associated with certain neutrophil functions. CD10 is a neutral endopeptidase that inactivates the chemotactic peptides and thereby prevents chemotaxis of neutrophils (7, 8). CD14 is a receptor for the complex of LPS and LPS binding protein (9), which increases in response to formyl peptide and LPS in inflammatory reactions (10). CD16 is an Fc receptor (Fc RIII) and directly associated with neutrophil activation (11). Normally, CD14 and CD16 are found together in secretory vesicles of neutrophils and when neutrophils are stimulated, CD14 and CD-16 co-migrate to the plasma membrane (12). It has also been shown that reduced expression of CD16 is a surrogate marker for apoptosis of neutrophils (13). Therefore, with these cell surface markers neutrophil functional profile can be evaluated more accurately by flow cytometry in a short time span.

Functional alterations of neutrophils of patients with BD, such as increased chemotaxis, adhesion and superoxide dismutase levels and decreased superoxide scavengering activity have been reported (14-18). In this study we aimed to investigate functional profile of neutrophils in patients with BD in comparison to inflammatory arthropathies, sepsis and healthy control subjects.

Materials and methods

Patients and controls

Oxidative burst, phagocytosis and surface molecules of neutrophils were investigated in a group of 50 patients (female/male: 19/31, age: 32 ± 9 years) with BD followed in the Behçet's Outpatient Clinic of Marmara Medical Faculty and classified according to the International Study Group Criteria (19). Sixty-six percent of patients (33/50) were HLA-B51 positive. At the time of the study, 26 out of 50 patients (52%) had at least one of the classical symptoms of the disease and classified as active cases. Twenty-nine patients (58%) were receiving first line (colchicine) and/or second line therapy (cyclophosphamide, azathioprine or thalidomide).

Twenty-nine patients with inflammatory arthropathies including 15 patients with rheumatoid arthritis (RA, female/male: 13/2, age: 44 ± 6 years), 14 patients with ankylosing spondylitis (AS, female/male: 2/12, age: 43 ± 12 years), and 15 patients with sepsis (female/male:6/9; age: 36 ± 12 years) were also investigated as inflammatory disease controls. A group of 53 healthy controls (female/male: 20/33, age: 31 ± 13 years) were also studied.

Oxidative burst

White blood cells (WBC) were obtained from heparinized peripheral blood samples by using erythrocyte lysing solution (155 mM NH_4Cl; 10 mM $KHCO_3$; 0.1 mM EDTA). Oxidative burst was evaluated using a previously described method (20). Briefly, 3-4 x 10^6 WBCs in PBS gel buffer (PBSG; PBS including %0.1 gelatin) were incubated with 20mM of dichlorofluorescin diacetate solution (DCFH-DA,

Kodak, Eastman) for 15 minutes at 37°C. After washing with PBSG, 1/3 part of WBCs was immediately evaluated flow cytometrically in order to define basal level of activation. Remaining 2/3s of WBCs were incubated with either 100 ng/ml of phorbol myristate acetate (PMA, Sigma) or 1 µg/ml of N-formyl-methyl-leucin peptide (fMLP, Sigma) 45 minutes at 37°C. After washing with PBSG, fluorescence alterations were immediately evaluated by flow cytometry (FACSort equipped with CellQuest software, Becton Dickinson, Mountain View, CA). Neutrophils were gated on two parameters cytogram, forward angle (FSC) vs 90° light scatter (SSC). DCFH-DA fluorescence was analysed on FL1 histogram with linear scale. Stimulation ratio was calculated as the ratio of mean value of fluorescence intensity of DCF-DA labelled neutrophils before and after stimulation with fMLP or PMA.

Oxidative burst in previously stimulated neutrophils

In order to test the oxidative burst in previously stimulated neutrophils, WBCs from a healthy control were first stimulated with PMA or fMLP at the same concentrations for 45 minutes at 37°C. Cells were then washed with PBSG and labelled with DCF-DA and the above mentioned procedure was followed.

Phagocytosis

Phagocytosis was measured by using a standard kit containing FITC-labeled *Esherichia coli* particles (Orphogen, USA) according to the manufacturer's instructions. Samples were evaluated with flow cytometry within 60 minutes. At least 10,000 cells were counted. Neutrophils were gated using two parameters (FSC vs SSC) and FL2 cytograms. Phagocytosis was analysed on FL1 histogram with log scale as a percentage of fluorescence. The sample incubated on ice was used as negative control.

Neutrophil phenotyping

Antibodies to CD10 (FITC), CD14 (PE), CD16 (FITC), CD45 (FITC), mouse IgG1 (FITC)/IgG2a (PE) isotypic controls were obtained from Becton Dickinson (Mountain View, CA). Cells were labeled with those antibodies by using standard immunostaining method and, analysed flow cytometrically within two hours. The neutrophil population was gated on the two parameter, forward angle (FSC) versus 90° light scatter cytogram (SSC). The percentage of cells expressing surface antigens of interest was determined by using apropriate isotypic controls.

Statistical analysis

Statistical analyses were performed by using SPSS software. Kruskal Wallis one way analyzes of variance test was used for comparisons. Mann Whitney-U test was used for post hoc corrections. All p values given were two-tailed.

Results

Oxidative burst

The stimulation ratio of neutrophils following PMA stimulation were 2.1 ± 1.1 in BD, 3.7 ± 2.8 in healthy controls, 4.0 ± 3.1 in inflammatory arthropathies and, 1.5 ± 0.2 in sepsis (Table I). Following fMLP stimulation, the ratios were 1.3 ± 0.5 in BD, 2.0 ± 1.7 in healthy controls, 1.7 ± 1.0 in inflammatory arthropathies and, 1.2 ± 0.3 in sepsis (Table I). The stimulation ratio of neutrophils following PMA or fMLP stimulations was found to be significantly decreased in patients with both BD and sepsis compared to healthy controls and inflammatory arthropathies ($p < 0.001$ and $p < 0.01$, respectively).

To test whether diminished production of oxygen radicals might be a consequence of previous stimulation of neutrophils, WBCs obtained from a healthy controls were stimulated twice, as mentioned in Methods, and the stimulation ratio of a second stimulation was evaluated. The stimulation ratios of previously stimulated neutrophils was lower than that of the unstimulated neutrophils, although the overall channel values were similar after PMA stimulation (Fig. 1).

When the stimulation ratio of neutrophils was evaluated in BD patients regarding the disease activation, treatment status and positivity of HLA-B51, there was no difference between active and inactive, treated and non-treated cases or according to HLA B51 status (Table I).

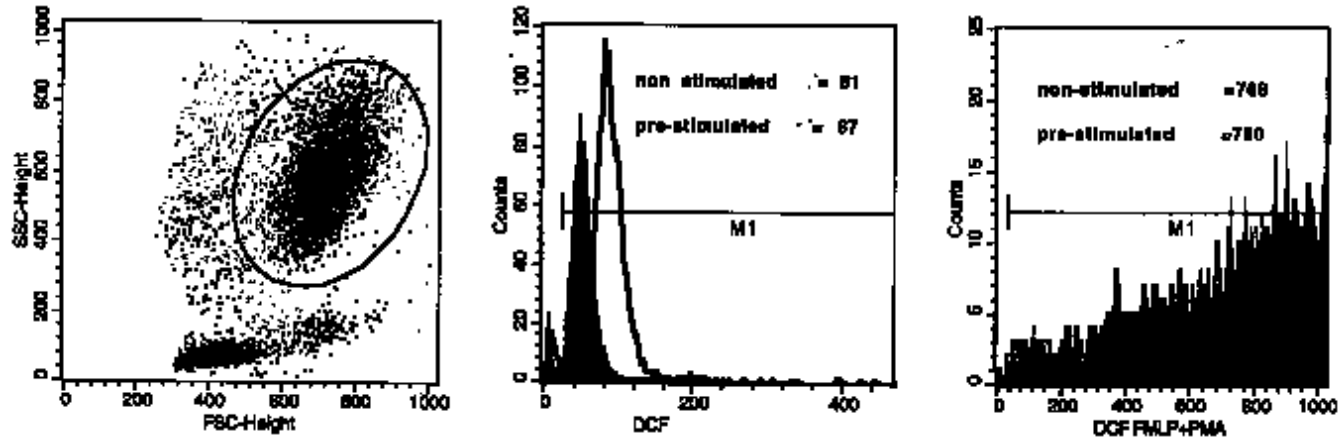

Fig. 1. A representative figure showing channel variations in unstimulated and FMLP pre-stimulated neutrophils. The channel value of an unstimulated sample was 51 in time 0, while this was 87 in fMLP pre-stimulated sample. Following PMA stimulation for 45 minutes, channel values of both sample reached similar numbers (768 in unstimulated and 780 in pre-stimulated) but stimulation ratios were 15 in unstimulated and 8.9 in pre-stimulated neutrophils.

Table I. The oxidative burst responses to PMA and fMLP stimulations in patients with Behçet's disease (BD), healthy controls (HC), inflammatory arthropathies (IA) and sepsis (SP) patients.

	N	Stimulation ratio following PMA (CI)	Stimulation ratio following fMLP (CI)
Behçet's disease	50	2.1 ± 1.1 (1.8-2.4)	1.3 ± 0.5 (1.2-1.5)
B51 (+)	16	2.6 ± 0.6 (1.8-2.4)	1.2 ± 0.4 (1.2-1.5)
B51 (-)	15	2 ± 1 (1.8-2.4)	1.3 ± 0.6 (1.2-1.5)
Healthy Controls	53	3.7 ± 2.8 (2.9-4.5)	2.0 ± 1.7 (1.4-2.5)
Inflammatory Arthropathies	29	4.0 ± 3.1 (2.7-5.4)	1.7 ± 1.0 (1.3-2.1)
Sepsis	10	1.5 ± 0.2 (1.3-1.7)	1.2 ± 0.3 (1.0-1.5)
P*		0.0001	0.01

CI: 95% confidence interval for mean, NS: non-significant.
*:P value given in the table is the result of comparison obtained by Kruskal Wallis,one way analyses of variance. Mann Whitney-U test:between BD and IA p = 0.001 for PMA,p = 0.02 for fMLP; between BD and HC p = 0.001 for PMA,p = 0.01 for fMLP; between BD and SP = 0.03 for PMA,NS for fMLP; between IA and HC NS for both; between IA and SP = 0.001 for PMA,NS for fMLP; between SP and HC p = 0.006 for PMA and p = 0.01 for fMLP.

Table II. The mean percentage of phagocytosis and mean fluorescence intensity (MFI) in patients with Behçet's disease, healthy and inflammatory control subjects.

	N	Phagocytosis ± SD (%) (CI)	MFI ± SD (CI)
Behçet's disease	37	81 ± 11 (77-85)	1226 ± 930 (916-1536)
B51 (+)	15	84 ± 13 (72-86)	1360 ± 802 (866-1728)
B51 (-)	11	91 ± 7 (79-90)	1136 ± 1308 (449-1536)
Healthy Controls	39	84 ± 8(82-87)	1354 ± 858 (1076-1633)
Inflammatory Controls	20	67 ± 17 (57-76)	1220 ± 405 (996-1445)
P*		0.001	NS

MFI:mean fluorescence intensity, CI: 95% confidence interval for mean,NS: non-significant. In Inflammatory control group there were 6 patients with RA, 5 with AS and 9 patients with sepsis. Since there were no significant differences between inflammatory arthropathies and sepsis these 2 groups were combined.
*:P value given in the table is the result of comparison obtained by Kruskal Wallis,one way analyses of variance. Mann Whitney-U test showed that the difference was stemmed from diseased controls (all p values obtained from post-hoc comparisons between diseased controls and others were smaller than 0.01).

Phagocytosis

The results of phagocytosis were summarized in Table II. The percentage phagocytosis of labelled *E. coli* particules in a given time interval was 81 ± 11% in BD, 84 ± 8% in healthy control subjects and 67 ± 17% in diseased controls. Neutrophil phagocytosis was significantly low only in diseased control subjects (p = 0.001, Table II).

The intensity of labelled particles (mean flourescence intensity, MFI) uptaken in a given time unit was not different between the three groups. Similarly, no effect of disease activation, HLA-B51 or treatment was observed on phagocytosis levels in BD group.

Phenotypic features of neutrophils

The percentage and the mean flouresence intensity values of neutrophil surface molecules were given in Table III. The surface density of neutral endopeptidase (CD10) was found to be significantly higher in patients with BD as well as in diseased controls (p < 0.001, Fig 2a). The mean percentage of CD14 expression, which is known to be LPS receptor was also higher in patients both with BD and diseased controls (p < 0.001, Fig 2b). The mean percentage of CD16 expression was low only in patients with sepsis (p < 0.001), whereas the intensity of CD16 on the cell surface was found to be low in both patients with BD and sepsis (p < 0.01, Fig. 2c). Increased CD14 expression and decreased surface CD16 intensity was observed in active patients compared to inactives (CD14: active: 25 ± 21% vs inactive: 10 ± 10%, p = 0.02; CD16: active: MFI: 317 ± 97 vs inactive: 467 ± 180, p = 0.05).

Discussion

In this study, neutrophil functions and cell surface markers were evaluated with flow cytometric methods in patients with BD compared to healthy and diseased controls.

Various studies showed increased neutrophil chemotaxis, O_2 release, enzymatic activity and phagocytosis in active BD compared to healthy controls (14-16, 21), but the results are conflicting. Mege *et al*. have shown increased basal O_2 production, but no differences in fMLP-stimulated neutrophils (14).

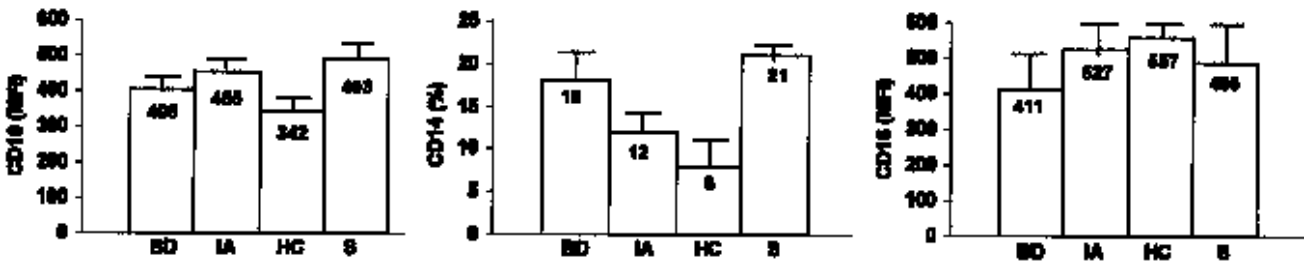

Fig. 2. The mean fluorescence intensities of CD10 and CD16 and mean percentage value of CD14 on neutrophils in patients with BD, healthy control subjects (HC),inflammatory arthropathies (IA) and patients with sepsis (S). Lines that extend from the box show the highest values of 95% of confidence interval of mean.

Table III. Cell surface markers in patients with Behçet's disease (BD), healthy controls (HC), inflammatory arthropathies (IA) and sepsis (SP) patients.

	N	CD10		CD14		CD16	
		mean % ± SD	MFI ± SD	mean % ± SD	MFI ± SD	mean % ± SD	MFI ± SD
Behçet's disease	35	96 ± 2.9	405 ± 35	18 ± 15	523 ± 69	97 ± 2.4	411 ± 99
Healthy controls	35	93 ± 7	341 ± 99	8 ± 12	501 ± 49	97 ± 3	558 ±41
I. Arthropathies	21	97 ± 2.5	455 ± 38	12 ± 10	498 ± 122	92 ± 9	527 ±166
Sepsis	15	95 ± 3.3	493 ± 24	21 ± 7	535 ± 201	81 ±16	486 ± 378
P*		NS	0.0001	0.001	NS	0.001	0.008

SD: standard deviation, MFI: mean fluorescence intensity, NS: non-significant.
*: P value given in the table is the result of comparison obtained by Kruskal Wallis, one way analyses of variance.
Mann Whitney-U test: for CD10 MFI: p = 0.007 between BD and HC; p = 0.0001 between BD and SP; BD and IA, HC and SP; HC and IA; p = 0.02 between IA and SP. For mean % of CD14: p = 0.009 between BD and HC; NS between BD and SP, and BD and IA; p = 0.0001 between HC and SP, HC and IA. For mean % of CD16:NS between BD and HC,BD and IA,HC and IA; p = 0.001 between BD and SP, HC and SP; p = 0.01 between SP and IA. For MFI of CD16:p = 0.004 between BD and HC; NS between BD and SP; HC and IA,IA and SP; p = 0.01 between BD and IA, p = 0.009 between HC and SP.

Pronai *et al*. observed increased O_2 both in stimulated or unstimulated neutrophils (15), whereas Takeno *et al*. observed increased production only after fMLP stimulation (21). Carletto *et al.*, on the other hand, found no changes in basal or stimulated O_2 production compared to controls (16). Similarly, Tüzün *et al*. observed no differences in chemotaxis compared to controls in both sexes (22).

In our study, decreased stimulation ratio of neutrophils in patients both with BD and sepsis after fMLP stimulation may indicate that neutrophils in both diseases were previously stimulated. As shown by the study of consecutive stimulations of neutrophils, re-stimulation of pre-stimulated neutrophils reveals less increase of O2 radicals production, suggesting a state of "pre-activation". More prominent decreases observed in patients with sepsis following PMA stimulation also supports this observation. An *in vivo* "primed" state of neutrophils with a dual signalling system for activating neutrophil oxidase is recently suggested (23). Agents such as fMLP, TNF- G-CSF or GM-CSF are shown to increase the tyrosine phosphorylation of various intra-cellular proteins and prime neutrophils *in vivo* without full activation. Levels of some of these pro-inflammatory cytokines such as TNF- are shown to be increased in BD sera and stimulated culture supernatants previously (14, 24).

In phagocytosis experiments, neutrophils from patients with BD had normal phagocytic function for the phagocytosis of labelled *E. coli* particles whereas decreased phagocytosis were observed in sepsis patients. Exhaustion of neutrophils with continuous hyper-stimulation probably caused this neutrophil dysfunction in sepsis patients in the end stage of severe inflammatory response.

The evaluation of neutrophil surface antigens also revealed significant changes in surface molecules associated with neutrophil activation and apoptosis in patients with BD and inflammatory controls. One of these molecules, CD10, is a neutral endopeptidase and is associated with neutrophil chemotaxis and priming of neutrophils for the release of lysosomal granules and production of superoxide (8, 25). CD14 is LPS receptor and is associated with neutrophil activation (9, 10,26). Monocyte CD14 expression and soluble CD14 was found to be elevated in BD sera previously (27). In this study, the expressions of CD10 and CD14 on neutrophil surface was also found to be elevated both in BD patients and inflammatory controls. Decreased expression of CD16 on neutrophils was similarly shown to be associated with neutrophil activation and apoptosis (28, 29). Morphologically apoptotic cells exhibit also CD16 low phenotype (13). In this study, the intensity of CD16 expression was also found to be decreased in patients with BD, suggesting a pathway to apoptosis. As expected, this change on neutophil surface was more prominent in patients with sepsis compared to BD.

The cause of neutrophil activation in BD is still unknown. A constitutional defect of neutrophils in association with HLA-B51 is suggested by Takeno *et al*., both in HLA-B51 positive BD patients and healthy controls, similar to HLA-B51 transgenic mice which have an increased oxidative burst response to fMLP (21). We could not confirm these observations as no differences were observed in neutrophil functions in our BD group according to HLA-B51 status. Alternatively, elevated levels of neutrophil activating cytokine and chemokines such as IL-6, IL-8 or TNF- might be responsible from the inflammatory mileu which keep BD patients neutrophils in a pre-activated, primed state continuously (14, 24, 30).

In conclusion, our findings strongly indicate that neutrophils in BD are active *in vivo*, the intensity of the activation is similar to some of the well known inflammatory conditions such as inflammatory arthropathies and sepsis. However these findings can not answer whether the type of activation is different from a standard inflammatory response seen in other inflammatory conditions such as infection or autoimmunity.

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