The effects of nitric oxide donors and inhibitors on neutrophil functions in Behçet's disease

G. Atalay[1], E. Eksioglu-Demiralp[2], T. Akoglu[2], H. Direskeneli[3]

Department of [1]Internal Medicine, [2]Department of Hematology-Immunology and [3]Department of Rheumatology, Faculty of Medicine, Marmara University, Istanbul, Turkey.

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Please address correspondence and reprint requests to: Haner Direskeneli, MD, Department of Rheumatology, Marmara University Hospital, Tophanelioglu Cad. 13/15, Altunizade, Istanbul, Turkey. E-mail: haner@marmara.edu.tr

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ABSTRACT

Objective. *The effects of nitric oxide donor SNAP and nitric oxide inhibitors L-NMMA and AG on the functions of neutrophils in patients with Behçet's Disease (BD) were investigated in vitro.*

Methods. *Oxidative burst and phagocytosis of neutrophils were evaluated by flow cytometry in patients with Behçet's disease (n = 32), inflammatory (n = 17) and healthy controls (n=14), in the presence of L-NMMA, AG and SNAP.*

Results. *The stimulation index of oxidative burst was found to be significantly decreased following PMA stimulation in patients with active BD compared to inflammatory and healthy controls. Oxidative function of neutrophils were inhibited in all 3 groups in the presence of L-NMMA, AG and SNAP. L-NMMA inhibited the oxidative burst of neutrophils obtained from healthy controls more than inflammatory controls and BD (80% vs 52% and 53% respectively, p = 0.001). No significant difference of phagocytosis inhibition was found with L-NMMA, AG and SNAP and there were also no differences between the groups (% 9-39).*

Conclusion. *Nitric oxide donors and inhibitors may have a therapeutic role in Behçet's disease by suppresing neutrophil activity.*

Introduction

Behçet's disease (BD) is a chronic, inflammatory vasculitis characterized by muco-cutaneous, ocular, vascular, arthritic and neurological involvement. The etiology and pathogenesis of BD is unclear, but various immunological abnormalities associated with both innate and adaptive immune systems have been reported (1). Activation of neutrophils such as increased respiratory burst, adhesion molecule expressions, superoxide dismutase levels and decreased superoxide scavenging activity have been reported in patients with BD (2-4).

The short-lived, highly reactive nitric oxide (NO) radical is implicated in many physiological roles, including vascular homeostasis and neuro-transmission. However, NO is also an important inflammatory mediator, produced by neutrophils and macrophages after stimulation with proinflammatory cytokines (5). The inhibition of induced NO synthase enzyme selectively by aminoguanidine (AG) or the production of NO by NO analog L-N monomethyl-L-Arginine (L-NMMA) may attenuate inflammatory responses (6). NO donors, like S-nitroso-N-acetylpenicillamine (SNAP) were also found to inhibit neutrophil functions in several studies (7).

The functional status of neutrophils can be evaluated by various methods, including the flow cytometric approach which is fast and simple (8). In this study, we aimed to investigate the changes in the phagocytosis and oxidative burst functions of neutrophils after incubating *in vitro* with L-NMMA, AG and SNAP in patients with BD.

Materials and methods

Patients and controls

Oxidative burst and phagocytosis of neutrophils were investigated in 32 patients (female/male: 13/19, mean age: 33 ± 13 years) with BD and compared with 17 healthy controls (HC) (female/male: 7/10, mean age: 29 ± 3 years) and 14 inflammatory controls (IC) (8 with pneumonia, 2 with systemic vasculitides and one each with pleurisy, sepsis, osteomyelitis and glomerulonephritis) (female/male: 7/7, mean age: 45 ± 12 years). All patients with BD fulfilled the International Study Group Criteria for Behçet's Disease (9) and were being followed in the out-patient Behçet's Clinics of Marmara Medical Faculty. Patients who had only muco-cutaneous involvement were considered to have

"mild" disease (n = 19) and they were receiving colchicine or non-steroidal anti-inflammatory drugs. Medications in these patients were stopped for one week before blood sampling. Patients who were receiving immunosuppressive therapy (corticosteroids, azathioprine, cyclophosphamide or cyclosporin-A) for major organ involvement (central nervous system, ocular or vascular disease) were considered to have "severe" BD (n = 13). Therapy was also stopped in these patients for a week, except immunosupressant drugs. The pathergy test was positive in 86% (24/28) and HLA-B51 in 83% (15/18).

Oxidative burst

Leucocytes isolated from 3 ml of peripheral blood were incubated with L-NMMA (20 l, from 1 mM solution), SNAP (100 l, from 10 mM solution) and AG (100 l, from 1 mM solution). After incubation at room temperature for 15 minutes, 1 l DCFH-DA was added to each tube for another 15 minutes and 100 ml of solution was analyzed by flow cytometry, as previously described (4). The remaining solution in each tube was incubated with 10 l PMA for 45 minutes at 37°C. Following stimulation with PMA, alterations in fluorescence were immediately evaluated by flow cytometry (FACSort equipped with CellQuest software, Becton Dickinson, Mountain View, CA). DCFH-DA fluorescence was analysed on FL-1 histogram with a linear scale. Stimulation indices (SI) was calculated as the ratio of the mean value of the fluorescence intensity of DCF-DA labelled neutrophils before and after stimulation with PMA.

Phagocytosis

Phagocytosis was measured using a standard kit containing FITC-labelled *Escherichia coli* particles (Orphogen, USA) according to the manufacturer's instructions. Thirty l of heparinized whole blood samples were incubated with L-NMMA, SNAP and AG for 10 minutes at room temperature. Then 7.5 ml of labelled *E. coli* particles were added to each tube and the negative control tube was placed on ice and the other tubes were placed in a 37°C water bath. After 20 minutes, first 25 l of quenching solution, then 1.5 ml of washing solution were added and the tubes were centrifuged for 5 minutes at 1500 rpm. Erythrocytes were then lysed. In the last step, cells were stained with 50 ml of DNA stain and samples were evaluated with flow cytometry within 60 minutes. Neutrophils were gated using two parameters (FCS vs SCC) and FL2 cytograms. Phagocytosis was analysed on FL1 histogram with a log scale as the percentage of fluorescence.

Statistical analysis

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

Results

Following PMA stimulation, no differences were observed between the stimulation indices of healthy (14.2 ± 6.0) and inflammatory controls (12.0 ± 7.6) and patients with mild BD (14.8 ± 8.4). However, the SI of patients with severe BD was significantly lower compared toallother groups(7.0 ± 3.4, $p < 0.005$). Oxidative burst of neutrophils was found to be inhibited in all 3 groups in the presence of L-NMMA, AG and SNAP, with a representative figure shown (Figs. 1 and 2). L-NMMA inhibited the oxidative burst of neutrophils obtained from healthy controls more than inflammatory controls and BD (80% vs 52% and 53% respectively, p = 0.001). The inhibition of oxidative burst was also found to be significantly higher in patients with severe BD compared to mild disease in the presence of L-

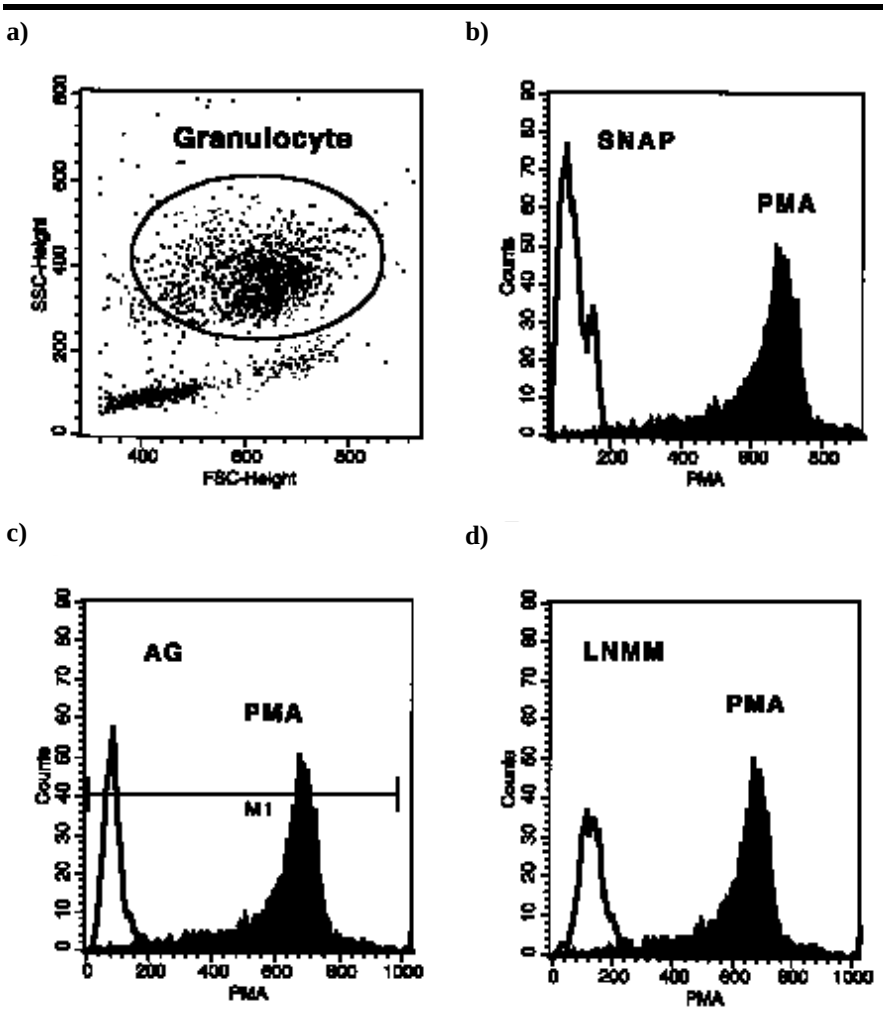

Fig. 1. A representative figure showing channel variations of neutrophils: (**a**) after PMA stimulations in the presence of SNAP (**b**) , AG (**c**) and L-NMMA (**d**) incubations.

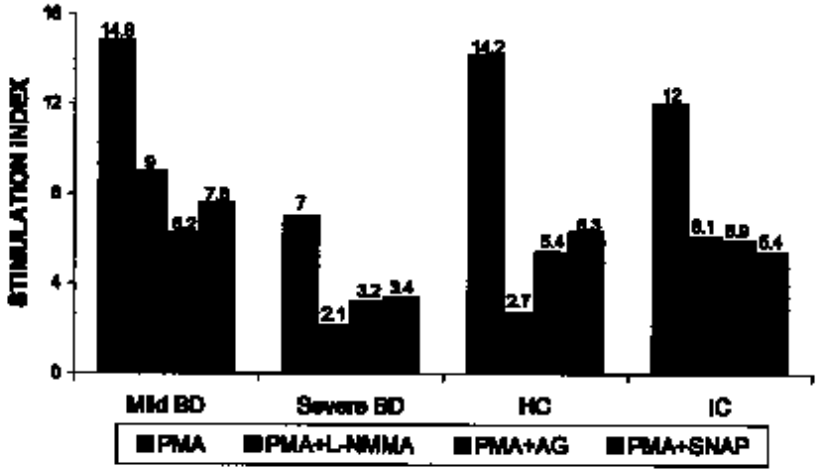

Fig. 2. Oxidative burst stimulation indexes in patients with BD (mild and severe),healthy and inflammatory controls after PMA stimulation in the presence of L-NMMA, AG, SNAP and PMA only. HC: healthy controls, IC: inflammatory controls.

NMMA (69 % vs 41 %, p = 0.04). Phagocytosis was also inhibited in all groups by L-NMMA, AG and SNAP (9-39%) (Fig. 2). No significant difference was found between the inhibition of phagocytosis in patients with mild and severe BD and between L-NMMA, AG and SNAP.

Discussion

Neutrophils express iNOS and cNOS and synthesize NO upon stimulation with PMA (10). We aimed to inhibit NO production in neutrophils by using a competitive inhibitor of the binding of L-Arginine to NOS, L-NMMA and an iNOS inhibitor aminoguanidine. L-NMMA inhibits iNOS more potently than e-NOS, whereas aminoguanidine reduces the formation of NO by either inhibiting iNOS protein expression or iNOS activity (11). Aminoguanidine has been found to be nearly equipotent with L-NMMA in its ability to inhibit NO production by stimulated macrophages, but less potent than L-NMMA on eNOS *in vivo.* Some studies have also reported the inhibition of neutrophil functions by NO releasing compounds such as SNAP (7), which increases extracellular NO by directly affecting NADPH oxidase (12).

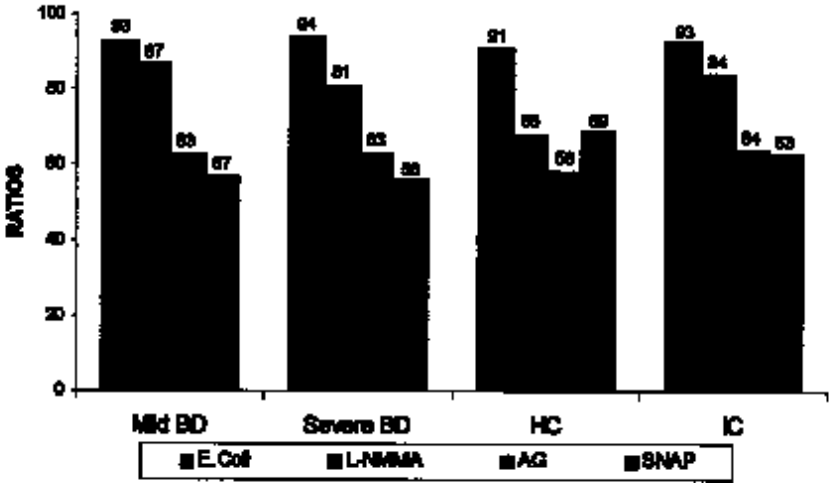

Fig. 3. Phagocytosis in patients with BD (mild and severe), healthy and inflammatory controls in the presence of L-NMMA, AG, SNAP and *E. coli* only. HC: healthy controls, IC: inflammatory controls.

In this study we observed that both the phagocytosis and oxidative burst of neutrophils from patients with BD and controls are suppressed in the presence of L-NMMA, AG and SNAP. It seems that L-NMMA inhibits the oxidative burst more effectively compared to other agents. This may be due to the fact that it is a competetive inhibitor of iNOS, and as a substrate inhibits its function earlier. Aminoguanidine acts during the induction phase of the enzyme and SNAP acts through the inhibition of NADPH oxidase, and their late mechanism of action may cause a lower inhibition of iNOS.

Neutrophils of patients with BD are suggested to be functionally abnormal due either to a constutitional defect, which might be linked to HLA-B51, or related to the increased amounts of cytokine and chemokines in the sera of these patients (1, 2). This inflammatory mileu might keep the neutrophils in a pre-activated state continuously and may explain the low level of stimulation indexes which we saw in severe cases compared to milder disease (4). However, the immunosuppressives which had to be continued in our severe cases might have also influenced the suppressed neutrophil responses. Higher suppressive effects of L-NMMA on healthy neutrophil burst responses compared to severe BD might also be related to the low baseline oxidative burst levels in these patients.

Given the increased Th1 cytokine profile and also the presumed endothelial dysfunction in BD, an increased synthesis of inflammatory and decreased synthesis of endothelial NO could be expected. Örem *et al.*, and Oksel *et al*. separately reported decreased plasma nitrate and nitrite levels in BD and suggested the presence of an endothelial dysfunction (13, 14). NO end products are also reported to be low in the tears of active BD patients with uveitis (15). However, as NO is an highly reactive and short lived molecule it is difficult to interpret the plasma levels without measuring NO synthetase activities.

Given its role as an effector molecule in inflammation, NO seems to represent an attractive potential target for pharmacological intervention in inflamma-

tory disorders. When given to patients with septic shock, L-NMMA or other inhibitors of NOS produce widespread vasoconstriction, consistent with the hypothesis that overproduction of NO contributes to the pathophysiology of septic shock. On the other hand, endothelial NO production is decreased in endotoxinemia, and since endothelially derived NO exerts a variety of benefical effects which are vasculoprotective in nature, NO donors also have been studied in endotoxic and related forms of circulatory shock syndrome with broad-based cytoprotective effects (16). However, further basic and clinical studies are required before the exact functions of NO and the therapeutic role of NO inhibition is clarified in BD and other inflammatory disorders.

In conclusion, although NO levels are reported to be low in BD, NO donors and inhibitors, possibly through iNOS inhibition, may play an anti-inflammatory therapeutic role by suppressing the oxidative burst and phagocytosis in Behçet's neutrophils.

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