Oxidative burst response to monosodium urate crystals in patients with Behçet's syndrome

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ABSTRACT

Objective. *An erythematous response to intradermal injection of monosodium urate crystals (MSU) has been demonstrated in Behçet's syndrome (BS). To further elucidate the pathogenesis of this response, the effects of MSU on* in vitro *oxidative burst reaction of neutrophils and monocytes were investigated.*

Methods. *Peripheral blood mononuclear cells from patients with Behçet's syndrome (BS), rheumatoid arthritis (RA), familial Mediterranean fever (FMF) and healthy controls (HC) were incubated with 100 ng/ml phorbol myristate acetate (PMA) and MSU at different dosages (25-500 μg/ml). Oxidative burst reaction was evaluated in neutrophils and monocytes by flow cytometry.*

Results. *In patients with BS, oxidative burst of neutrophils was significantly increased compared to HC at 125 μg/ml and 250 μg/ml dosages of MSU ($p \leq 0.001$ and 0.004 respectively). In patients with FMF, there was also an increased oxidative burst reaction at 75 μg/ml, 250 g/ml and 500 μg/ml ($p \leq 0.007$; 0.001 and 0.004 respectively). In patients with BS, oxidative burst of monocytes was increased only at 125 g/ml dosage of MSU ($p \leq 0.002$). However, in patients with FMF monocyte burst response was increased at 25 μg/ml, 75 μg/ml and 125 g/ml ($p \leq 0.004$; < 0.0001; < 0.0001 and 0.002 respectively). In RA group, stimulation with PMA resulted in a higher oxidative burst reaction than FMF and BS ($p \leq 0.000$ and $p \leq 0.008$). No correlation was observed between oxidative burst of neutrophils or monocytes and intradermal responses to MSU crystals.*

Conclusion. *Oxidative burst reaction with MSU is augmented in neutrophils and monocytes of BS. However, the response is not specific and is unassociated with skin dermal test which has a high specificity for BS.*

Introduction

Behçet's syndrome (BS) is a multisystemic vasculitis characterized by recurrent oral aphthae, genital ulcers, mucocutaneous lesions and uveitis. The most prominent immunopathology of BS is the augmented non-specific inflammatory response of which skin pathergy reaction is a classical example. A Th1-type, proinflammatory response results in increased chemotaxis, superoxide formation and adhesion molecule expression in BS (1-5). Stimulation of neutrophils by formyl-methyl-leucin-peptide (fMLP) causes an increase in superoxide formation in comparison to healthy controls which suggests the role of *in vivo* stimulated neutrophils in the pathogenesis (1, 5).

In assessing the oxidative burst in neutrophils, the most widely used standard methods measure the extracellular release of superoxide anion or hydrogen peroxide by their ability to reduce or oxidize substrates (ex. dichlorofloresc in diacetate solution; DCFH) to colored or fluorescent products. Upon activation, membrane associated NADPH-oxidase produce superoxide and hydrogen peroxide which consequently oxidates the substrate to give green or red fluoresence (6).

In gouty arthritis, phagocytosis is the main mechanism of inflammation caused by monosodium urate (MSU) crystals. The type of the cell that phagocytes the MSU crystal has a critical role in this inflammatory process: The encounter of MSU crystals with monocytes or neutrophils results in an inflammatory reaction, while no such reaction occurs with macrophages (7, 8, 9). Local and systemic inflammatory reaction provoked by MSU crystals has led to the idea of urate skin test in BS. Cakir *et al*. has first shown that skin reaction provoked by intradermal injection of MSU crystals resulted in an increased erythematous response in

patients with BS in comparison to healthy and diseased controls and concluded that the skin test with MSU crystals is more sensitive than classical pathergy reaction in BS (10). Fresko *et al*. have recently reported the sensitivity and the specificity of the urate skin test as 75% and 76%, respectively (11). In order to clarify the mechanism of local dermal reaction of MSU crystals, *in vitro* stimulation of neutrophils and monocytes with MSU crystals was investigated in this study in BS patients and controls.

Table I. Mean area of the urate skin test and disease duration in patients with BS and control groups (± SD).

	F/M	Age (year) ± SD years	Area of urate skin test ± SE mm^2	Disease duration ± SD years
Behçet's	15/15	35.5 ± 8.3	467.2 ± 105.0*	10±6.2
RA	11/3	45.2 ± 11.9	14.1 ± 9.7	9.5±3.6
FMF	9/11	29.8 ± 8.8	103.5 ± 57.1	16.7±7.2
Healthy control	10/10	33.8 ± 8.3	83.9 ± 69.1	_

*p < 0.001: BS vs control groups.

Materials and methods

Patient and control groups

Thirty patients with BS classified according to the International Study Group Criteria and followed in the multi-disciplinary Behçet's out-patient clinic in Cerrahpasa Medical Faculty were included in the study. All BS cases had clinically mild, mucocutaneous disease, followed up without medication. Nineteen patients with familial Mediterranean fever (FMF), 14 with rheumatoid arthritis (RA) and 20 healthy controls (HC) were also studied as control groups. Patients with FMF were without attacks and patients with RAhad no active arthritis at the time of the study. Informed consent was obtained from all participants and the study was approved by the local ethical committee. Characteristics of the patient and control groups are shown in Table I.

Preparation of urate crystals

Urate crystals were prepared by a modification of Seegmiller's method (12). Pure uric acid (1.68 g) was added to 400 ml of boiling distilled water and adjusted to pH 8 with 0.5 N NaOH. This supersaturated solution was kept at room temperature for 24 hours to allow the crystals to precipitate. The crystals were then heated at 180°C for 3 hours. Immediately before use, the required amount of crystals to be injected was suspended in 0.2 ml sterile normal saline, briefly sonicated, and checked by polarised light microscopy.

Urate skin test

2.5 mg of urate crystal suspension was injected intra-dermally into the flexor surface of the non-dominant forearm. At 48 hours the erythema that developed was marked on a paper and the area was measured in mm^2. Urate skin test was performed in 8 healthy controls, 13 patients with RAand in all patients with BS and FMF.

Oxidative burst response

White blood cells were obtained from heparinized peripheral blood samples by using erythrocyte lysing solution (155 mM NH4Cl, 10mM KHCO3, 0.1 nM EDTA). 3-4 x 10^6 white blood cells in PBS gel buffer (PBSG; PBS including %0.1 gelatin) were incubated with 20 mM dichloroflorescin diacetate solution (DCFH-DA, Kodak, Eastman), for 15 minutes at 37°C. After washing with PBSG, 1/7 part of white blood cells was immediately evaluated flow cytometrically in order to determine basal level of activation (FACSort, CellQuest software, Becton Dickinson, Mountain View, CA). The remaining 6/7 white blood cells were incubated at 37°C with 25 µg/ml, 75 µg/ml, 125 µg/ml, 250 µg/ml, 500 µg/ml of MSU and 100 ng/ml of phorbol myristate acetate (PMA). Fluorescence changes in monocytes and neutrophils were evaluated by flow cytometry. Due to technical reasons florescence changes in monocytes were evaluated in 29 patients with BS, 13 patients with RA, 18 patients with FMF and 20 healthy controls. The stimulation ratio was calculated for monocytes and neutrophils separately as the ratio of chemiluminicense before and after stimulation.

Statistical analysis

All statistical analysis were performed by SPSS 8.0. Comparisons between groups were performed by Kruskal Wallis and Mann-Whitney-U tests. Pearson

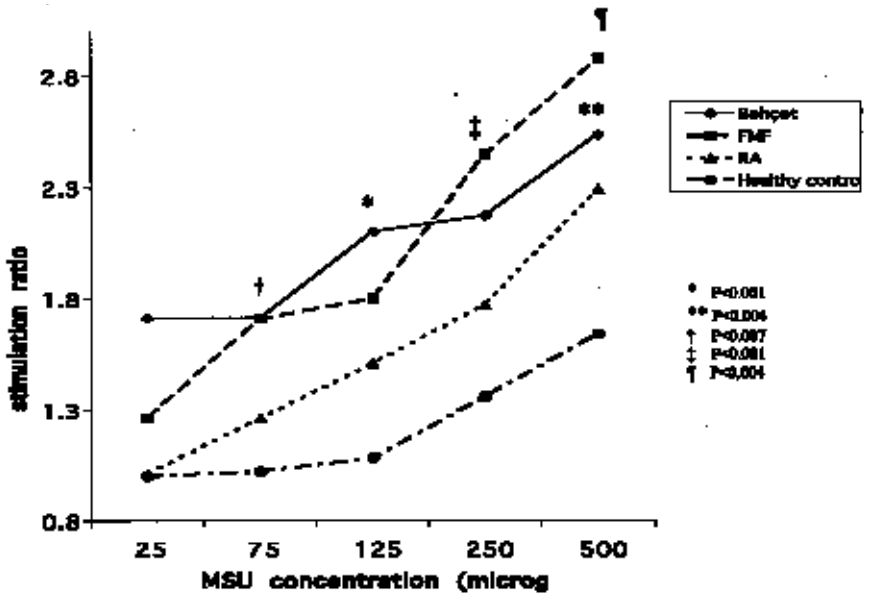

Fig. 1. Dose response curve in neutrophils in patients with BS and control groups.

Table II. Mean and 95% confidence interval values of stimulation ratios with different concentrations of MSU crystals and 100ng/ml PMAin neutrophils.

	25 µgr/ml MSU mean (95%CI)	75 µgr/ml MSU mean (95%CI)	125 µgr/ml MSU mean (95%CI)	250 µgr/ml MSU mean (95%CI)	500 µgr/ml MSU mean (95%CI)	100 ng/ml PMA mean (95%CI)
Behçet	1.3 (1 – 1.5)	1.7 (0.9 – 2.6)	2.1 (1 – 3.2)	2 (1.6 – 2.6)	2.4 (1.9 – 2.9)	4.8 (3.6 – 6)
FMF	1.2 (0.9 – 1.6)	1.7 (1.3 – 2.1)	1.8 (1.3 – 2.3)	2.5(2 – 3)	3 (2.1 – 3.9)	3.8 (2.1 – 5.5)
RA	1.1 (0.9 – 1.4)	1.3 (1.3 – 1.6)	1.5 (1 – 1.9)	1.8(1.1 – 2.4)	2.3 (1.5 – 3)	1.8 (1.2 – 2.5)
Healthy control	1 (0.8 – 1.2)	1 (0.8 – 1.2)	1 (0.9 – 1.3)	1.4(1 – 1.7)	1.6 (1 – 2.3)	3.3 (2.2 – 4.4)

Table III. Mean and 95% confidence interval values of stimulation ratios with different concentrations of MSU crystals and 100ng/ml PMAin monocytes.

	25 µgr/ml MSU mean (95%CI)	75 µgr/ml MSU mean (95%CI)	125 µgr/ml MSU mean (95%CI)	250 µgr/ml MSU mean (95%CI)	500 µgr/ml MSU mean (95%CI)	100 ng/ml PMA mean (95%CI)
Behçet	1.3 (1 – 1.5)	1.4 (1.2 – 1.6)	1.7 (1.3 – 2.1)	2 (1.6 – 2.4)	2.2 (1.8 – 2.7)	2 (1.6 – 2.5)
FMF	1.6 (1.1 – 2)	2.2 (1.6 – 2.8)	2.4 (1.8 – 3)	3 (2.2 – 3.7)	3.4 (2.1 – 4.7)	2.4 (1.7 – 3)
RA	1.2 (0.9 – 1.6)	1.5 (1 – 1.9)	1.7 (1.1 – 2.3)	2 (1.3 – 2.6)	2.5 (1.5 – 3.5)	1.3(1.1 – 1.5)
Healthy control	0.9 (0.8 – 1.1)	1 (0.8 – 1.2)	1 (0.8 – 1.3)	1.5 (1 – 2)	1.7 (1 – 2.4)	1.3(0.9 – 1.8)

test was performed for the correlation between the oxidative burst response, disease duration and the area of erythema of urate skin test.

Results

Urate skin test

The area of erythema provoked by MSU was larger in Behçet's syndrome than that of the diseased and healthy controls ($p < 0.001$) (Table I).

Oxidative burst response of neutrophils

The basal neutrophilic activity did not differ between Behçet's syndrome and control groups ($p = 0.26$). Oxidative response was increased in patients with BS in comparison to HC at 125 µg/ml and 500 g/ml concentrations of MSU ($p \leq 0.001$ and 0.004, respectively). In patients with FMF, oxidative response was increased in comparison to HC at 75 µg/ml, 250 µg/ml and 500 µg/ml concentrations of MSU ($p \leq 0.007$; 0.001 and 0.004, respectively). No difference was observed between the patients with BS, FMF and RA (Fig. 1, Table II). In RA group, stimulation with PMA resulted in a higher oxidative burst reaction than FMF and BS ($p \leq 0.000$ and $p \leq 0.008$). Oxidative burst reaction with either MSU or PMA did not correlate with the area of urate skin test (r= -0.01, -0.08, -0.06, -0.02, -0.00, 0.19 respectively for MSU crystal concentrations of 25 µg/ml, 75 µg/ml, 125 µg/ml, 250 µg/ml, 500 µg/ml and 100 ng/mlPMA) or disease duration (r = –0.03, -0.13, -0.16, -0.08, -0.09, -0.03 respectively for MSU crystal concentrations of 25 µg/ml, 75 µg/ml, 125 µg/ml, 250 µg/ml , 500 µg/ml and 100 ng/ml PMA).

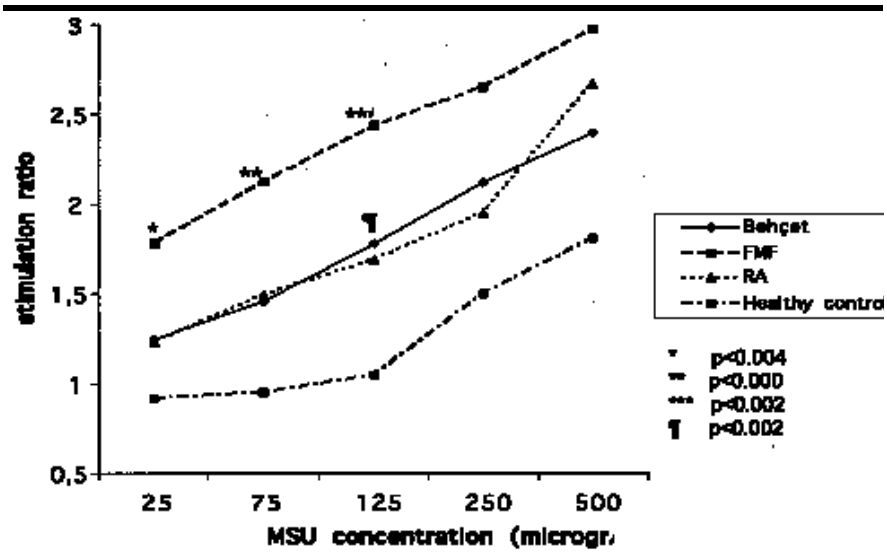

Fig. 2. Dose response curve in monocytes in patients with BS and control groups.

Oxidative burst response of monocytes

The basal oxidative burst response of monocytes was increased in patients with RA compared to HC ($p \leq 0.004$). In patients with Behçet's syndrome at 125 µg/ml MSU the oxidative burst response was increased in comparison to HC ($p \leq 0.002$) (Fig. 2, Table III). In the FMF group there was an increased burst response at 25 µg/ml, 75 µg/ml and 125 µg/ml concentrations of MSU ($p \leq 0.004$; 0.000; 0.002 respectively). Stimulation with PMA resulted in an increased response only in patients with FMF in comparison to HC ($p \leq 0.003$). Oxidative burst reaction of monocytes with either MSU or PMA also did not correlate with the area of urate skin test (r = 0.06, -0.04, -0.003, –0.004, 0.08, 007 respectively for MSU crystal concentrations of 25g/ml, 75 µg/ml, 125 µg/ml, 250 µg/m, 500 µg/ml and 100 ng/ml PMA) or disease duration (r =

0.08, 0.03, 0.04, 0.02, 0.14, -0.06 respectively for MSU crystal concentrations of 25 µg/ml, 75 µg/ ml, 125 µg/ ml, 250 µg/m, 500 µg/ml and 100 ng/ml PMA)

The monocytic and neutrophilic burst reactions with MSU correlated with each other. This correlation was prominent at 25 µg/ml, 250 µg/ml and 500 µg/ml concentrations of MSU (r = 0.71, 0.76, and 0.79 respectively; p values for the correlations were = 0.00, 0.00, 0.00 respectively). Oxidative burst response between neutrophils and monocytes with PMA had a modest correlation (r = 0.58).

Discussion

In this study oxidative burst response to MSU in monocytes and neutrophils in BS compared to healthy and diseased controls was investigated. There was an increased response to MSU crystal stimulation in both neutrophils and monocytes in BS. However this response was not specific as similar increases were also observed in patients with FMF. Our results have shown a general correlation between *in vitro* neutrophilic and monocytic burst reactions, but these responses did not correlate with the urate skin test. Similar to previous studies, urate skin test was observed to be highly sensitive for BS in our study (10, 11).

Studies of neutrophilic oxidative burst reaction in BS have shown conflicting results (3, 14, 15). Enrollment of patients with different disease activities or treatment regimens might have influenced these discrepancies. Colchicine and steroids have been shown to decrease neutrophilic functions (15, 16). Eksioglu-Demiralp *et al*. have reported a lower ratio of oxidative burst in patients with BS and suggested that the already *in vivo* stimulated neutrophils could not further be stimulated *in vitro*. However, BS population in this study consisted of patients with a variable clinical spectrum and 65% of them were on colchicine or immunosuppressive drugs (13). In our study, basal oxidative response in both neutrophils and monocytes of patients with BS has shown no difference compared to healthy controls. Patients with BS in our study had mainly mucocutaneous involvement and were followed up without medication. Seven patients had a history of eye involvement but they were void of uveitis attacks for a long time. This milder clinical course may explain similar basal oxidative burst reactions in our study in BS compared to healthy controls.

As it is generally accepted that clinical activity of BS decreases with disease duration, one may speculate that the neutrophilic activity would wane as the activation of the disease wanes. However, in our study, oxidative burst response in both neutrophils and monocytes did not correlate with disease duration suggesting that the decrease in disease activity in BS could be due to mechanisms other than the activity of neutrophils and monocytes.

Although no data is available about the immunopathology of the urate skin test, the pathology of the pathergy test has been well characterized. An extensive infiltration of mononuclear cells consisting mainly of monocyte/macrophages and T cells have been documented (17-19). There are conflicting reports about the role of neutrophils in pathergy reaction, but along with monocytes, neutrophils play an important role in the inflammation provoked by MSU (19). In this study no correlation could be detected between neutrophil or monocyte oxidative responses and the area of erythema of the urate skin test. The pathophysiology of the urate skin test in BS may involve a complex process in which not only neutrophils and monocytes but other elements of innate and acquired immunity, such as macrophages and T-cells might be involved. A generalized immune defect is suggested in BS by Hirohata *et al*. in a pivotal study showing increased interferon-γ response of T-cells to low-dose superantigens (20). Another possibility is the role of cytokines such as IL-12 and IL-18, secreted by neutrophils after stimulation by Th1 cells and further stimulate monocytes. In the study by Sahin *et al.*, CD14, a marker of monocytic activation, was found to be raised in patients with BS (21).

Neutrophilic oxidative responses were also found to be elevated in FMF. Anton *et al*. have reported increased neutrophilic oxidative response to fMLPin FMF patients who are void of attacks (22). In patients with FMF, the oxidative burst reaction in monocytes was also more prominent than neutrophils. The defective expression of pyrin also in monocytes associated with MEFV mutations can explain this observation (23). Although the most prominent cell type in FMF is neutrophils, the presence of monocytes has also been detected in the synovial fluid of patients with arthritic involvement (23).

In the RA group, although a statistically higher difference was observed in basal monocyte response only, both the neutrophil and monocyte basal responses were found to be the highest among study groups. Similarly Crocker *et al*. have reported a higher oxidative response to fMLF in non-pregnant RA patients in comparison to healthy controls (24). These findings are not surprising as RA is characterised by an accumulation and activation of leucocytes, predominantly neutrophils.

In conclusion, MSU crystals provoke an oxidative burst response in both neutrophils and monocytes of BS patients. Although this burst reaction is not specific, our findings suggest the prominent role of innate immunity in the pathogenesis of BS. However, the discrepancy between positivity of the urate skin test and *in vitro* innate responses suggests the requirement of additional factors for urate skin responses, possibly associated with acquired T-cell associated immunity. Further studies on the mechanisms of urate intake into antigen-presenting cells and the activation of adaptive immunity in BS is warranted.

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