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Neutrophil adhesion to endothelial cells and factors affecting adhesion in patients with Behçet's disease

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Abstract

***Objectives*—To study the in vitro adhesion of polymorphonuclear leucocytes (PMNLs) to endothelial cells in patients with Behçet's disease (BD), and the humoral and cellular factors which may contribute to adhesion.**

***Methods*—A total of 118 patients with BD and 60 healthy controls were studied. In vitro adhesion of chromium-51 labelled normal neutrophils to human umbilical vascular endothelial cell (HUVEC) monolayers were studied in the presence of normal serum or serum obtained from patients with BD. Adhesion of neutrophils from patients with BD to HUVEC stimulated with tumour necrosis factor (TNF), interleukin-1 (IL-1), and lipopolysaccharide (LPS) and adhesion molecule (CD11a, CD11b, CD18 and L-selectin) expression on the patient's neutrophils and lymphocytes were determined, and the serum concentration of IL-8 was measured.**

***Results*—Sera from patients with BD were found to enhance the adherence of normal PMNLs to HUVEC monolayers in vitro. Patients' sera induced an increase in surface expression of CD11a and CD18 on normal neutrophils and intercellular adhesion molecule-1 (ICAM-1) expression on HUVECs. The number of CD11a positive neutrophils was greater in the blood of patients with BD than in that of healthy controls (89·4% *v* 71%; p < 0·001). Pretreatment of HUVECs with IL-1α, TNFα or LPS resulted in an increased adhesion of patients' PMNLs greater than that observed for normal PMNLs. Monoclonal antibodies to CD11a, CD11b, CD18, and ICAM-1 caused varying degrees of inhibition of neutrophil adhesion. The concentration of IL-8 was also found to be significantly increased in sera of patients with BD (490 (SD 470) pg/ml) compared with normal controls (97·5 (56·3) pg/ml).**

***Conclusion*—Abnormalities of neutrophils, endothelial cells, or both, have been suggested to be responsible for many of the clinical manifestations of BD. Our findings may explain the underlying mechanism of neutrophil accumulation in Behçet's lesions.**

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Behçet's disease is a multisystemic inflammatory disorder affecting many organs, the major sites of involvement being the eyes, skin, and mucous membranes. The widespread lesions seen in the early stages of inflammation are characterised microscopically by intense polymorphonuclear leucocyte (PMNL) infiltration—an appearance that has stimulated research into PMNL and endothelial cell function in these patients. Chemotactic activity of PMNLs in patients with Behçet's disease has been reported to be high,[1] with increased leucocyte migration in vivo.[2] Incubation of normal leucocytes with neutrophil supernatants obtained from patients with Behçet's disease leads to an augmented chemotactic response in the normal leucocytes,[3] and the phagocytic activity of neutrophils from these patients has also been reported to be increased.[1 4] Although acid phosphatase and β-glucuronidase activities are normal in patients with this disease, myeloperoxidase activity of neutrophils has been found to be decreased just before ocular attacks, and then to increase gradually.[5] All these findings indicate the presence of activated neutrophils in patients with Behçet's disease.

Abnormalities in endothelial cells, such as defective 6-keto prostaglandin-F1 production and increased thromboxane B2 levels also have been reported in patients with Behçet's disease,[6] though the role of endothelial cells in the inflammation is not clearly known.

A number of inflammatory mediators have been found to be increased in the sera of these patients. In addition to various antibodies to cellular components (H Direskeneli *et al*: Proceedings of the XIIth European Congress of Rheumatology, Budapest, 1991), concentrations of tumour necrosis factor (TNF), soluble CD8, and interleukin-2 (IL-2) receptor were high in the serum of these patients.[7] Monocytes obtained from patients with Behçet's disease were reported to produce increased amounts of IL-6 and IL-8 in in vitro cultures.[8] IL-1 concentrations were also found to be increased in the serum of these patients.[9] Many of these substances are known to affect both endothelial cells and neutrophils.

We have investigated adhesion of neutrophils to normal and stimulated endothelial cells in patients with Behçet's disease, and studied various adhesion molecules and mediators that may contribute to neutrophil adhesion.

Materials and methods

PATIENTS AND CONTROLS

A total of 118 patients satisfying the International Study Group criteria for Behçet's disease[10] were investigated throughout the study. Seventy eight of them had active disease (presence of at least one major symptom) and 40 had inactive disease at the time of study. The healthy control group consisted of a total of 60 normal laboratory personnel and medical students. The number of patients and controls included in different experiments varied. Fifteen millilitre of venous blood for serum or 5 ml of heparinised (5 U/ml) venous blood for neutrophil isolation (or both) were obtained from each patient and control. Sera were divided into aliquots and stored at –40°C until required for use.

PREPARATION OF NEUTROPHILS FOR ADHESION ASSAY

Human peripheral blood PMNLs from five healthy individuals were isolated in one step from whole blood after centrifugation through lymphoprep (Nycomed, Norway) as described by Ferrante and Thong,[11] and pooled. Residual erythrocytes were removed by hypotonic saline lysis and cells were suspended in serum free RPMI medium. The PMNL yield was more than 95% and viability, assessed by the ethidium bromide-acridine orange dye exclusion method, was more than 95%. Neutrophils were then radiolabelled with chromium-51 according to the method of Gamble *et al.*[12]

PREPARATION OF ENDOTHELIAL CELLS

Human umbilical vascular endothelial cells (HUVECs) were isolated from four or five umbilical cords as described by Jaffe *et al*,[13] pooled, and grown in RPMI-1640 medium (RPMI containing 20% fetal calf serum (FCS); Seralab, UK) with the addition of heparin 5 U/ml (Organon Teknika, Holland), endothelial cell growth factor (ECGF) 50 μg/ml (Sigma Chemicals Co, St Louis, MO, USA), 0·225% sodium bicarbonate, sodium pyruvate 1 mmol/l, L-glutamine 2 mmol/l, fungizone 2·5 μg/ml, and gentamycin 30 μg/ml. After three passages, an enzyme linked immunosorbent assay (ELISA) test for von Willebrand factor (Biolab, Belgium) was performed on culture supernatants in order to confirm the endothelial origin of the cultured cells. HUVECs used in the adhesion assays had undergone at least three, and no more then eight, passages.

ADHESION ASSAY

This was performed by the method described by Gamble *et al*,[12] with modifications. HUVECs were harvested as described by Jaffe *et al*[13] and distributed to 0·1% gelatin coated flat bottom 96 well plates ($1{\cdot}5 \times 10^4$ cells per well in 100 μl of medium). After overnight incubation, wells were washed gently with prewarmed (37°C) medium, care being taken not to remove cells from the wells. To each well, 50 μl of ^{51}Cr-labelled pooled normal neutrophil suspension (containing 75 000 cells) was added, then 50 μl of each patient or control serum was added to three wells. Plates were incubated for 90 minutes at 37°C in 7% carbon dioxide and wells were gently washed twice with phosphate buffered saline (PBS) to remove non-adherent neutrophils. Remaining cells (HUVECs and adherent neutrophils) were lysed by overnight incubation with 100 μl of 1% triton-X in PBS. Lysates were removed and counted in a gammacounter.

Adherence was determined as the percentage of the total radioactivity added to the wells (adherence ratio).

All patient and control sera were tested in triplicate. To obtain a background count, three wells were processed as the same way but without addition of PMN.

NEUTROPHIL ADHESION TO STIMULATED HUVECs

Confluent HUVECs in 96 well plates were washed twice with prewarmed (37°C) RPMI medium without ECGF and heparin, then 100 μl of RPMI containing lipopolysaccharide (LPS) 1 μg/ml (Sigma Chemical, USA), TNFα 10 ng/ml (Seralab, UK), or 10 U/ml of IL-1 (Seralab) was added for stimulation. Plates were incubated at 37°C for four hours and washed gently with PBS. Neutrophils obtained from five patients and five healthy controls were pooled separately, labelled with chromium-51, and the neutrophil adhesion assay performed as described above using stimulated HUVECs, or unstimulated HUVECs as control. All pooled sera were tested in 12 wells and results were expressed as the mean of the 12 wells.

BLOCKING OF ADHESION BY MONOCLONAL ANTIBODIES

HUVECs and normal pooled PMNLs obtained from five individuals were pretreated with monoclonal antibodies (MAb) for 15 minutes, following which the adhesion assay described above was performed in the presence of MAb. However, in contrast to the above experiments, five patient sera having a high adhesion capacity (at least one SD above the mean capacity of the patient group) were pooled and used in the adhesion assay. HUVECs were incubated with anti-E-selectin (clone ENA-2, F(ab)2 fragment, Monosan, The Netherlands) and anti-intercellular adhesion molecule-1 (anti-ICAM-1) (clone 15.2, F(ab)2 fragment, Cimbus Bioscience Ltd, UK). Neutrophils were incubated with anti-CD11a (clone MHM-24, Dakopatts AS, Denmark), anti-CD11b (clone-94, Coulter Electronics Ltd, UK), anti-CD18 (clone MHM-23, Dakopatts AS, Denmark), or combinations of these monoclonal antibodies. As a control, anti-CD19 (Coulter Electronics) was used to demonstrate that non-specific blockage of adhesion by mouse serum did not occur. Adhesion inhibition was calculated by the formula of Vennegor *et al.*[14]

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All combinations were tested in triplicate and six independent experiments were performed with the same patients' pooled serum. Results are presented as the mean of the six experiments.

SPONTANEOUS SURFACE EXPRESSION OF ADHESION MOLECULES

Five millilitre of venous blood samples was obtained from 30 patients with Behçet's disease and 30 normal individuals, mixed with 0·1 ml of 0·13 mol/l EDTA and 0·03 mol/l sodium azide solution, and immediately placed into an ice cold water bath. Blood samples (100 μl) were then incubated with 10 μl of monoclonal antibodies (anti-CD11a, clone MHM24, Dakopatts, AS, Denmark; anti-CD11b, clone 94, Cimbus, UK; anti-CD18, clone MHM23, Dakopatts, AS; anti-L-selectin, clone Dreg 56, Immunotech, France) for 30 minutes at 4°C and washed with ice cold PBS containing 0·1% bovine serum albumin and sodium azide 7 mmol/l. Further incubation for 30 minutes with 50 μl of 1/200 diluted fluorescein isothiocyanate labelled goat antimouse Ig (affinity purified, Serotec, UK), was followed by washing in cold PBS. Erythrocytes were then lysed with lysing solution (Becton and Dickinson, USA), and leucocytes were washed with the above buffer, fixed with 1% paraformaldehyde in PBS, and examined by flow cytometry (FACScan, Becton and Dickinson, USA). Patient and control samples were assayed at the same time with the flow cytometer at the same setting. Lymphocytes and neutrophils were gated separately and percentages of stained cells were estimated.

EFFECT OF PATIENT SERA ON ADHESION MOLECULE EXPRESSION

To examine the effects of patient serum on surface adhesion molecule expression, 50 patient sera were tested with normal PMNLs obtained from healthy laboratory personnel. Fifty microlitres of PMNL suspension (containing $0 \cdot 3 \times 10^6$ cells) was incubated with 50 μl of patient serum, normal control serum, or PBS alone for 90 minutes at 37°C. Cells were then washed twice with the wash buffer, stained by monoclonal antibodies as described above and analysed by using FACScan. Mean fluorescence intensities were also estimated, in order to measure the overall effect of patient serum on PMNLs.

The effect of patient serum on HUVEC surface adhesion molecules was also determined. Five patient sera with high adhesion capacity (at least one SD above the mean capacity of the patient group) were selected. HUVECs in 96 well plates were first washed with prewarmed medium, then 50 μl of medium without FCS and 50 μl of patient or control serum was added. Plates were incubated at 37°C in 7% carbon dioxide for 90 minutes, following which wells were washed twice with wash buffer. Cells were detached mechanically by pipetting, stained with monoclonal antibodies (anti-ICAM-1, clone 15.2, F(ab)2 fragment, Cimbus Bioscience Ltd, UK; anti-E-selectin, clone ENA-2, F(ab)2 fragment, Monosan, The Netherlands) as described above, and then analysed using FACScan. A total of 10×10^3 cells were assessed and antigen expression was presented as the relative mean fluorescence intensity.

DETERMINATION OF IL-8

IL-8 was determined in patient sera by ^{125}I-IL-8 competition radioimmunoassay (RIA) (Advanced Magnetic Inc, USA), following the manufacturer's instructions.

STATISTICAL ANALYSIS

Statistical differences between controls and patients were assessed using the Mann-Whitney U test and Student's unpaired t test as indicated in the text, and reported as the mean (SD). The relationship between clinical manifestations and laboratory findings was examined by regression analyses.

Results

ADHESION OF NEUTROPHILS TO HUVECs

Pooled neutrophils from healthy individuals showed significantly increased adhesion to HUVECs in the presence of Behçet's patients' sera compared with adhesion in the presence of normal serum. Table 1 shows the different numbers of individual sera obtained from patients with Behçet's disease and tested in seven different experiments. In each case, mean adhesion was always increased compared with control experiments conducted with normal serum. Because different HUVEC preparations were used in each experiment, there were some differences in the mean adhesion ratios obtained on each occasion (table 1), and a large SD. When all values from 118 patients obtained from seven experiments were assessed together (fig 1) and active and inactive cases separated, there was a significant difference between patients and normal controls, but no difference was observed between active and inactive cases.

Table 1 Adherence ratio of pooled normal neutrophils to human umbilical vascular endothelial cells in the presence of normal serum and serum obtained from patients with Behçet's disease

Experiment No§	*Patient sera†*		*Normal sera*		*p**
	Adherence ratio‡	*Number of sera*	*Adherence ratio‡*	*Number of sera*	
1	24·5 (11·7)	26	13·7 (11)	14	0·007
2	10·4 (2·5)	25	7·2 (3·5)	15	0·000
3	29·4 (6·5)	20	27·7 (3)	18	0·7
4	16 (14)	20	10·1 (6·4)	12	0·04
5	9·9 (3)	20	5·4 (1·5)	11	0·03
6	22·4 (7·8)	50	16·7 (5·7)	10	0·02
7	25·4 (11·8)	28	19·3 (7·8)	26	0·0008
Mean	19·7 (7·6)		14·3 (7·7)		0·01

Values are mean (SD).
†Tested individually in triplicate and mean obtained.
‡Calculated by dividing cpm of bound polymorphonuclear leucocyte lysates by total cpm and multiplying by 100.
§Experiments 1–5: total of 40 sera tested in different batches; experiments 6, 7: all sera tested once.
*Mann-Whitney U test.

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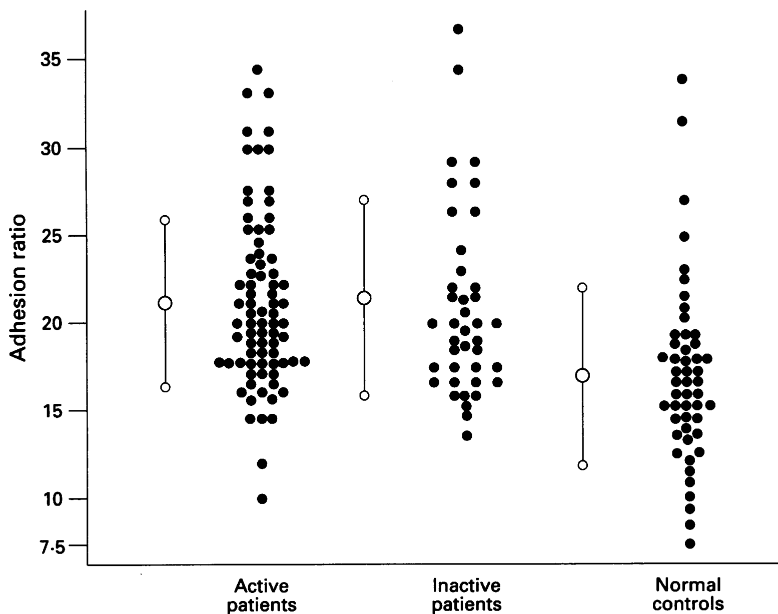

Figure 1 Neutrophil adhesion to human umbilical vascular endothelial cells in the presence of serum obtained from patients with Behçet's disease (n = 118) or from normal controls (n = 60), tested in seven different experiments. The first 40 patients' sera were tested more than once in five batches, and the mean of each patient's sera is presented in the figure; the remaining 78 sera were tested only once.

Stimulation of HUVECs by LPS, TNFα, and IL-1α caused increased adhesion of both normal and patient neutrophils. As expected, this effect was more pronounced for patient neutrophils (table 2).

EXPRESSION OF SURFACE ADHESION MOLECULES

The number of PMNLs expressing CD11a was significantly increased in patients (89·4 (10·8)% (n = 50)) compared with controls (71·0 (8·9)% (n = 19)). The proportion of cells expressing CD11b, CD18, and L-selectin was greater than 90% in both patients' and control PMNLs. Adhesion molecule expression on the lymphocytes also did not differ significantly in the patients with Behçet's disease compared with controls.

After incubation of normal neutrophils with sera from patients with Behçet's disease, the expression of CD11a and CD18 molecules increased markedly, whilst there was no increase in the expression of the CD11b molecule as measured by flow cytometry and assessed as the relative mean fluorescence intensity (table 3).

Figure 2 shows the expression of ICAM-1 on the endothelial cells after incubation with patient sera. Relative mean fluorescence intensity of the ICAM-1 (CD54) expression on endothelial cells was not significantly increased after treatment with patient sera, but the number of endothelial cells expressing ICAM-1 was slightly increased (greater peak value) after incubation (fig 2). Expression of E-selectin, however, did not change after incubation with patient sera.

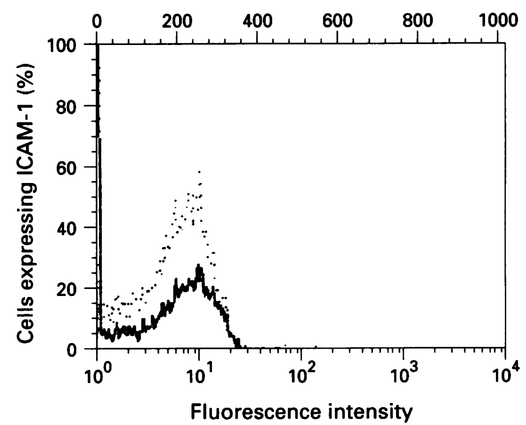

Figure 2 Relative fluorescence intensity of intercellular adhesion molecule 1 on human umbilical vascular endothelial cells after incubation with normal sera (—) or sera obtained from patients with Behçet's disease (......).

INHIBITION OF ADHESION BY MAb

All monoclonal antibodies against cell adhesion molecules tested in this study caused significant inhibition of normal neutrophil adhesion to HUVECs in the presence of patients' sera (table 4). This inhibition was greater with anti-CD11a and anti-CD18 treatment (37·5% and 37·6%, respectively) than that observed

Table 2 Adherence ratio to stimulated human umbilical vascular endothelial cells (HUVEC) obtained with polymorphonuclear leucocytes (PMNLs) from normal controls (n = 5) or from patients with Behçet's disease (n = 5)

	Normal HUVEC (%)	*HUVEC stimulated with*		
		IL-1α (%)	*TNFα (%)*	*LPS (%)*
Normal PMNL	18·5 (5·7)	37·8 (13)	35 (10·2)	31·4 (13·2)
Behçet's PMNL	23·6 (5·6)	61·5 (20·8)	57·4 (17·5)	55 (8·1)
p*	0·22	0·05	0·05	0·01

Values are mean (SD).
IL-1α = Interleukin-1α 10 U/ml; TNFα = tumour necrosis factor α 10 ng/ml; LPS = lipopolysaccharide 1 μg/ml.
*Mann-Whitney *U* test.

Table 3 Effect of sera from patients with Behçet's disease and normal control sera on adhesion molecule expression on normal polymorphonuclear leucocytes (PMNLs) (fluorescence intensity)

PMNLs treated with	*Expression of*		
	CD11a	*CD11b*	*CD18*
Normal sera	4·6 (6·3) (n = 10)	25·8 (21·3) (n = 10)	12·7 (13·9) (n = 10)
Patients' sera	20·5 (28·3) (n = 50)	20·9 (21·3) (n = 50)	28·1 (13·9) (n = 50)
p*	0·03	0·2	0·004

Values represents net mean fluorescence intensities (SD), calculated by substracting fluorescence intensities of PMNLs incubated with medium only (basal level), from the fluorescence intensities of PMNLs incubated with patients' sera or normal control sera.
*Mann Whitney-*U* test.

Table 4 Inhibition of polymorphonuclear leucocyte (PMNL) adhesion to human umbilical vascular endothelial cells (HUVEC) by monoclonal antibodies

Monoclonal antibodies	*Inhibition (%)*
CD19 (control)	0·6 (0·8)
CD11a	37·5 (4·5)
CD11b	20·2 (7·5)
CD18	37·6 (4·6)
ICAM-1	22·7 (9·1)
E-selectin	4 (4·4)
CD18 + CD11a	34·1 (10)
CD18 + CD11b	40·9 (5·7)
CD18 + CD11a + E-selectin	32·8 (14·2)
CD18 + CD11a + ICAM-1	34·1 (5·1)
CD18 + CD11b + E-selectin	38·4 (5·7)
CD18 + CD11b + ICAM-1	35·9 (12·1)

Values represents the mean (SD) of six independent experiments using different batches of HUVEC and different pooled normal PMN.
ICAM-1 = Intercellular adhesion molecule 1.

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using CD11b. Combination of these monoclonals produced synergistic effects and even greater inhibition. Anti-CD19 (a monoclonal antibody unrelated to adhesion molecules) was used as a control in the adhesion assay: no inhibition of adhesion was demonstrated (table 4).

SERUM CONCENTRATIONS OF IL-8

Mean serum concentrations of IL-8 were significantly increased in sera from patients with Behçet's disease compared with sera from normal controls (490 (470) pg/ml *v* 97·5 (56·3) pg/ml; p = 0·001). Fourteen sera from patients expressed greatly increased IL-8 levels (at least 2 SD greater than the mean). Eleven of these sera were also found to promote greatly increased adhesion ratio (adhesion ratio at least 2 SD greater than the mean). There was, however, no significant difference between active and inactive cases in terms of serum IL-8 concentrations. Furthermore, in those patients with increased serum concentrations of IL-8 there was no correlation between IL-8 values and adhesion ratios.

CORRELATIONS OF CLINICAL MANIFESTATIONS WITH INCREASED ADHESION

The increased neutrophil adhesion in vitro and increased serum concentrations of IL-8 in the patients with Behçet's disease were subjected to regression analyses, but there was no statistically significant correlation that might indicate clinical relevance of the findings.

Discussion

Behçet's disease is characterised histopathologically by neutrophilic vasculitis with increased neutrophil infiltration of the perivascular area.[15] A number of investigators have found abnormalities in neutrophil functions, such as increased chemotaxis and phagocytosis,[1] and abnormal neutrophilic infiltration in the pathergy reaction.[16] Circulating immune complexes have been found to be significantly increased in the serum of patients with Behçet's disease,[16 17] and have been suggested to be responsible for the vessel damage and enhanced neutrophil migration.[18]

Jorizzo *et al* tested neutrophil migration in a subagarose test system and showed increased migration of both Behçet's and normal neutrophils in the presence of serum from patients with Behçet's disease, but not with normal control serum.[18] They suggested that an unidentified, heat stable serum factor was responsible for enhanced migration. In the present study we also found enhanced adhesion of neutrophils to endothelial cell monolayers in the presence of serum from patients with Behçet's disease (table 1). In all seven independent experiments, the mean adhesion ratios obtained with patients' sera were increased compared with those for normal control sera. As observed in all experimental systems using cell culture, day to day differences between experiments was significant and standard deviations were high. However, as an increase in adhesion ratio was a consistent finding in all our experiments (table 1), we suggest that the increased adhesion found with sera from patients with Behçet's disease reflects the actual situation in this disorder.

The increased neutrophil adhesion in patients with Behçet's disease that we observed, and the significant increase of adhesion after stimulation of HUVECs by IL-1α, TNFα, and LPS (table 2), may reflect functional or quantitative upregulation of surface adhesion molecules on PMNLs in Behçet's disease. Indeed, the expression of some adhesion molecules on PMNLs obtained from patients with Behçet's disease was found to be quantitatively increased.

The number of neutrophils expressing lymphocyte function associated antigen 1 (LFA-1) (CD11a/CD18) molecules was increased in patients with Behçet's disease. Incubation of normal neutrophils with patients' sera induced a significant increase in CD11a and CD18 molecules (table 3), and MAbs against these molecules caused significant inhibition of adhesion compared with that observed in the presence of other MAbs. These findings indicate that the LFA-1 molecule, which is composed of CD11a and CD18, has a significant role in the increased adhesion of neutrophils from patients with Behçet's disease, and that E-selectin is not involved in the process. As inhibition of adhesion with different combinations of MAbs was not complete, the role of other adhesion molecules such as P-selectins should also be considered.

Interestingly, sera of patients with Behçet's disease caused a significant increase of in vitro adhesion and induced greater expression of adhesion molecule on normal PMNLs, which may indicate that an unknown serum factor(s) is responsible for all these abnormalities, as suggested previously by Jorizzo *et al.*[18]

A number of serum factors may be involved in the adhesion of PMNLs to endothelial cells: for example IL-1, IL-2, TNF, granulocyte macrophage colony stimulating factor, IL-6, platelet activating factor. Increased concentrations of IL-1, IL-6, and TNFα in the sera from patients with Behçet's disease have been reported previously.[9 19] Activated endothelial cells synthesise a signalling molecule, known previously as neutrophil activating factor (NAF) or neutrophil activating peptide 1 (NAP-1) and now identified as IL-8. This cytokine activates PMNLs by binding to its specific receptor, and upregulates β2 integrins.[20] In contrast, Gimbrone *et al* have demonstrated a completely opposite effect of IL-8:[21] they have shown that activated HUVECs secrete a soluble leucocyte adhesion inhibitor (LAI). Further studies have revealed that LAI activity is a function of the IL-8 produced by endothelial cells. It is now generally accepted that IL-8 has a dual function, and that in the microenvironment of inflamed tissue, IL-8 regulates neutrophil adhesion. In severe systemic conditions such as septic shock, a significantly increased plasma IL-8 concentration may protect the vessel wall from

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PMNLs mediated injury, through an adhesion inhibitory effect.[22]

The significance of the increased IL-8 concentrations present in the sera of patients with Behçet's disease remains open to speculation. Although we do not know the underlying mechanism for the increased plasma IL-8 in these patients, it may, together with IL-1, IL6, and TNF, be responsible for the increased adhesiveness of PMNLs found in this study. Alternatively, the increased IL-8 in the sera of our patients may have been a secondary phenomenon, the main function being protection of vessel walls from PMNL mediated injury, as suggested by Gimbrone.

We believe that these abnormalities reflect the inflammatory nature of Behçet's lesions, though these in vitro studies are far from representing the actual situation in the inflammatory microenvironment in the disease. Further studies of the other adhesion molecules and a more specific understanding of the interactions of different mediators and cell surface molecules will clarify the situation in patients with Behçet's disease.

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