In vivo leucocyte migration in Behçet's syndrome

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SUMMARY   Serial studies of leucocyte migration in vivo were carried out in 15 patients with Behçet's syndrome using a skin window technique. Where possible, patients with and without active disease were studied during and in the absence of treatment. In patients with active disease neutrophil migration was frequently greater than normal, particularly with respect to numbers of cells migrating. There was also an increased frequency of emigrating neutrophils with less or more nuclear lobes than normal. In three patients in whom function of skin window neutrophils was studied nitroblue tetrazolium reduction and phagocytosis and killing of *Candida guilliermondiae* were normal. The monocyte component of the skin window was more often reduced in patients than in normal controls. Corticosteroid treatment did not exert a major effect on leucocyte migration, though the doses involved were relatively small. Neutrophil abnormalities were common in patients and particularly those with active disease. These results suggest that neutrophil hyperactivity may have an important role in the pathogenesis of Behçet's syndrome.

Key words: white cell function, multisystem disease.

Behçet's syndrome is a multisystem disease, the major features of which are oral and genital ulceration, eye and skin lesions. It is thought primarily to have an immunological basis. Two of the more curious dermatological manifestations of Behçet's syndrome are the papular and pustular skin lesions which develop at sites of venepuncture[1][2] and the skin hyperreactivity to needle puncture—that is, pathergy reaction.[3] Histopathological evidence suggests that the underlying pathogenesis of Behçet's syndrome is a vasculitis, in which either mononuclear or polymorphonuclear leucocytes (neutrophils) predominate depending on the age of the lesion.[4][5]

A number of abnormalities of leucocyte function have been found and, in particular, increased neutrophil locomotion and chemotaxis in vitro have been demonstrated.[6–9] It has been suggested that the marked cellular inflammatory response which characterises Behçet's syndrome may be due to increased neutrophil locomotion,[6–8] as well as to possible abnormalities in neutrophil metabolism.[9]

These features render the skin of particular interest and strongly implicate leucocytes in the pathogenesis of Behçet's syndrome. We therefore studied leucocyte migration in vivo in 15 patients

with Behçet's syndrome using a skin window technique employing micropore membranes.[10]

Patients and methods

Fifteen patients with Behçet's syndrome (10 male, five female) aged 24 to 49 years (mean 32 years) were followed up serially over a period of 18 to 24 months. Seven were English, two Greek, two Indian, one Egyptian, one Iranian, one Spanish, and one Turkish. All patients had at least three of the four major criteria used in the diagnosis of Behçet's syndrome.[11][12] All 15 patients had a past or current history, or both, of oral ulceration, 13 (87%) genital ulceration, 11 (73%) uveitis, and 10 (67%) skin manifestations. Four patients had a history of developing papular or pustular skin lesions, or both, at venepuncture sites, but only one patient had a positive pathergy skin test.

Blood tests included a full blood picture, white cell count and differential, and erythrocyte sedimentation rate. Patients were studied, where possible, in four clinical states—namely, with or without active clinical disease and during or in the absence of treatment. For the purpose of this study active disease was defined as clinically detectable disease in one or more major or minor site, and inactive disease as no clinically detectable disease in any site.

Nine patients were studied with and without active disease while receiving no treatment and eight patients were studied during and in the absence of treatment. Treatments included prednisolone in eight patients (5–30 mg daily), cyclophosphamide in two (100 mg daily), and stanozolol in two (10 mg daily). No patient was taking colchicine at the time of study. All patients gave their full informed consent.

The skin window technique used in this study has been fully described elsewhere,[10] but a brief resumé is given here. Saline moistened micropore membranes (8 μm pore size) were placed on each of two superficial abrasions of forearm skin made with a dental burr. On these were placed in turn a dialysis membrane, a saline soaked filter paper pad, and a thick film of plastic to retain moisture. This assembly was taped to the arm and a tubular support bandage slipped over all. The first membranes on the arm were left in place for four hours, replaced by fresh membranes for 20 minutes, and these in turn were replaced by a third set for 60 minutes. The leucocyte infiltrated micropore membranes were then fixed, stained, and cleared for microscopic analysis. The second set of membranes (those on for 20 minutes) were used to assess neutrophil migration and the third set (those on the arm for 60 minutes) for monocyte migration. This last set was stained for non-specific esterase activity, which differentiates monocytes from other leucocytes.[13] In addition, neutrophils which had traversed the micropore membrane and adhered to the distal dialysis membrane were examined in three ways: (*a*) stained with a haematological reagent, Diff-Quick (American Hospital Supplies), for nuclear counts; (*b*) exposed to nitroblue tetrazolium (Sigma) and phorbol myristic acetate (Sigma) before being fixed and stained with 1% Safrannin and examined for nitroblue tetrazolium reduction[14]; and (*c*) exposed to opsonised yeasts (*Candida guilliermondiae* plus 10% fresh plasma) and fixed and stained with Diff-Quick and examined for phagocytic and killing activities.[14]

The following measurements were used for assessing the response in the micropore membranes: (*a*) neutrophil cellularity, which was a visual estimate of the relative degree of cellular infiltration in the 20 minute membranes and taken to be an indication of the number of cells emigrating; and (*b*) the leading front, which was the furthest point reached by cells in the 20 minute membranes and measured as the distance in micrometres from the starting surface— that is, that next to the skin. This gave an index of the rate of locomotion of the emigrating cells—that is, penetration of the membrane. To assess the neutrophil cellularity a ×40 field objective was used and the mean cell count of five different sites

calculated. Normal cellularity was defined as 15 to 30 cells per ×40 field, reduced cellularity as less than 15 cells per ×40 field, and increased cellularity as more than 30 cells per ×40 field.

Normal values were derived from the original description of the technique[10] and adjusted in the light of results obtained with clinically normal, age matched controls tested during the course of the study. Abnormal results in patients obtained during active and inactive disease were compared statistically using the sign test.[15] All subjects gave their informed consent and the study was approved by the committee on ethics of clinical investigation at the University College Hospital London School of Medicine.

Results

Neutrophil cellularity and leading fronts did not behave as independent variables, and it was rare to find a high value for the leading front in a membrane containing few cells or a low value in those containing many. The most common abnormality was greater neutrophil cellularity or leading front measurements, or both, than those obtained for normal subjects, particularly in patients with active disease (Fig. 1). Six patients (6/15, 40%) on seven occasions (7/34, 21%) showed an increase in neutrophil cellularity, on six occasions during active disease and on one occasion during inactive disease ($p<0.05$). Nine patients (9/15, 60%) on 12 occasions (12/34, 35%) showed an increase in leading front measurements, on nine occasions during active disease and on three occasions during inactive disease ($p<0.05$). In four patients studied on six occasions (four inactive, two active) neutrophil cellularity and leading front measurements were reduced. Indeed in two of these patients on three occasions (two inactive, one active) there was a striking diminution of the response. The mean (SD) white cell count in the patients' blood during active disease was 9.1 (2.7)$\times10^9$/l (range $5.1–14.8\times10^9$/l). Peripheral white cell counts below 15.0×10^9/l have had little effect on skin window neutrophil cellularity or leading fronts in any of the groups of patients we have studied so far.

Neutrophil nuclear lobe counts were made in the micropore membranes and, where possible, from the cells attached to distal dialysis membranes. The presence of more than an occasional (>1%) single lobed ('band form') or five and six lobed nucleus was considered abnormal, and this occurred on 13/22 (59%) occasions. Generally there was an increase in the range of lobe counts, with multilobed cells being the most common abnormality (Table 1). In one patient with active disease, however, 16%
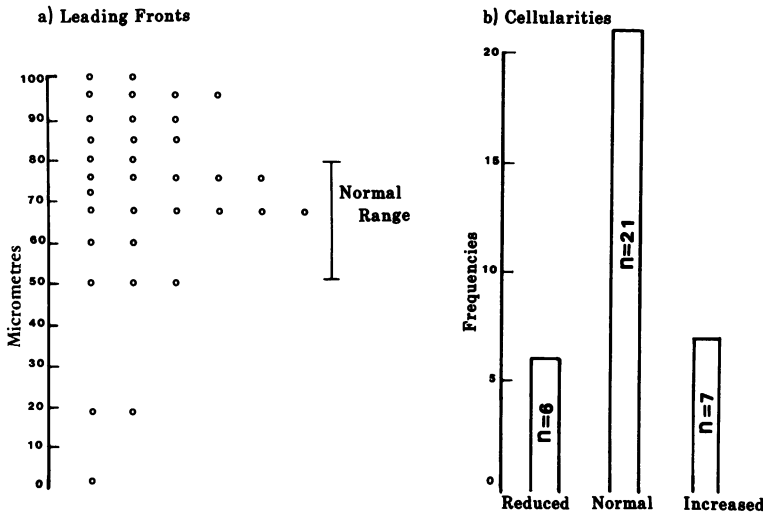
a) Leading Fronts

b) Cellularities

Fig. 1 *Leading fronts and cellularities (see text) were assessed in membranes left on the arm for 20 minutes, at the height of the response, in 15 patients studied on a total of 34 different occasions. No patient contributed more than three results.*

Table 1 *Nuclear lobe counts in skin window neutrophils from 15 patients with Behçet's syndrome*

Range of lobe counts	Frequency (No of occasions)
2 to 4 (normal)	5
2 to 6	5
1 to 4	6
1 to 6	6

Where possible, nuclear lobes were counted in neutrophils which had entered micropore membranes or traversed them and adhered to the distal dialysis membrane. The 15 patients were studied on a total of 22 different occasions, and no patient contributed more than two results to any category.

band forms were seen, together with five and six lobed cells. None of the normal subjects had >1% band forms or five or six lobed nuclei. Thus the picture overall showed an increase in the range of neutrophil forms relative to normal subjects, particularly in patients with active disease.

Monocyte cellularity and leading fronts were measured as for neutrophils but in terms of non-specific esterase positive cells.[13] Unlike neutrophils, monocytes appeared to show a variation in the numbers emigrating (cellularity) and in the rate of further movement (leading front), which were independent of each other. For example, a patient

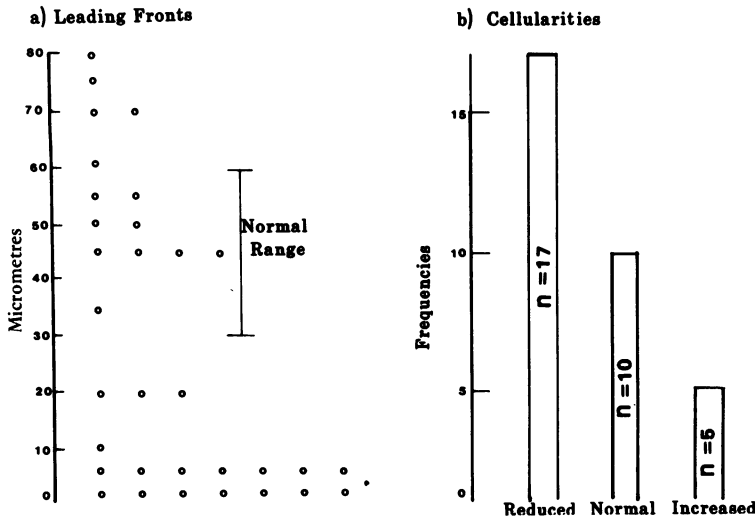a) Leading Fronts

b) Cellularities

Fig. 2 *Monocytes were recognised as cells staining diffusely by the non-specific esterase technique. Cellularity includes responses where there was emigration onto the lower surface of the membrane but little or no migration into the membrane itself. The 15 patients were studied on a total of 32 different occasions, and no patient contributed more than two results.*

with active disease on one occasion showed an increased cellularity and a low leading front at the same time. On most occasions, however, the monocyte response was reduced in terms of both parameters (Fig. 2), though there was no clear cut association with disease activity (Table 2). In addition, there were no significant differences in the results of neutrophil or monocyte tests between those patients with ocular manifestations and those with orogenital ulcers and skin lesions.

In three patients with active disease neutrophils attached to the distal dialysis membrane were tested for reduction of nitroblue tetrazolium and phagocytic activity. The reduction was positive in all cells—that is, was normal in all three patients. After 60 minutes' incubation with opsonised yeast, cell associated yeast particles were seen, about 20% of which stained in a manner indicating that they had undergone intracellular degradation. Although as yet we have insufficient experience with these preparations to be conclusive about their normality, the results do suggest that emigrant cells retain some degree of normal function in these patients despite the frequency of abnormal forms.

Sequential studies showed that changes in cellular responses occurred equally often when a patient

Table 2 *Frequency of abnormal findings in nine patients tested during active and inactive disease*

	Active	Inactive
Neutrophil abnormalities		
Nuclear morphology	8	3
Emigration*	6	1
Penetration†	9	3
Monocyte abnormalities		
Emigration	6	3
Penetration	6	4

The table is compiled from sequential data showing the frequency of abnormal results during active and inactive disease in nine patients in whom it was possible to study leucocyte function in both of these clinical states.
*Emigration refers to cellularity in the skin window membranes—that is, the degree of cellular infiltration.
†Penetration refers to the leading front—that is, the distance travelled from the starting surface on the skin.

Table 3 *Frequency of changes in skin window responses shown by nine patients tested sequentially*

	Same clinical state	Change of clinical state
Change in neutrophil response	6/7	6/9
Change in monocyte response	6/9	5/7

Patients were followed up serially so that sequential tests were made either while patients remained in the same clinical state or while spanning a change. The table records the number of times a marked difference in response occurred in each of these situations.

appeared to be stable within one of the four clinical states—that is, with or without active disease and during or in the absence of treatment, as when they had changed from one state to another between tests (Table 3). When abnormalities were listed against clinical state at the time of testing (Table 2) it was evident that they were more common during active disease.

Discussion

Neutrophil cellularity (emigration) or penetration (leading front), or both, in skin window membranes was frequently greater than normal in patients with Behçet's syndrome, particularly during active disease. These findings are in agreeement with the in vitro findings of increased neutrophil activity previously reported.[6-9] Sobell et al found both increased neutrophil migration and increased serum chemotactic activity,[6] while Abdulla and Lehner found only increased serum activity.[7] Fordham et al observed increased cellular mobility only, particularly in the numbers of cells moving.[8] Of particular relevance to our results are those of James et al,[16] whose in vitro studies of neutrophil migration showed a decreased response, in contrast with both our in vivo results and with their own. They used a fluid chamber skin window technique, however, which, as discussed elsewhere,[10] may give a negative image of neutrophil activity. Thus more active cells being more adherent are less likely to detach themselves to appear free in the chamber fluid.

Our findings particularly resemble those of Fordham et al,[8] who emphasised the increase in the number (rather than individual activity) of neutrophils entering micropore membranes in vitro. This, taken together with the abnormal range of neutrophil forms seen among the emigrant cells, suggests that an element of dysregulation of neutrophil supply, maturation, or life span, may be present in Behçet's syndrome. This conclusion is similar to that reached by Wilton and Lehner, who postulated that a proportion of circulating neutrophils were immature in this disease.[9]

Neutrophil abnormalities were more common in patients with active disease, supporting the idea that neutrophil hyperactivity may play some part in the pathogenesis of the disease. The marked changes in response (to be expected in such an episodic disease), however, did not always occur during a period of obvious clinical change. Indeed, they were seen as frequently during stable periods as when the patient changed from one clinical state to another between tests. Although this appears to argue against an active role for altered neutrophil responsiveness in Behçet's syndrome, this may equally be

the result of a delay between biological change in the patient and detectable clinical change. Two patients showed a striking diminution in neutrophil cellularity and penetration, apparently unrelated to disease activity or immunosuppressant treatment, and the explanation for this is not clear.

Treatment, and in particular corticosteroids, did not appear to exert a major influence on leucocyte migration or other leucocyte functions, though our numbers were too small to be certain of this. It is relevant here that previous work using the same skin window technique, in patients with systemic lupus erythematosus, detected only a minor decrease in neutrophil response after much larger doses of corticosteroids.[17]

Despite the increased frequency of neutrophils with more or less lobes to their nuclei than is normal the limited studies of nitroblue tetrazolium reduction, phagocytosis, and killing performed on cells which had penetrated the membranes suggested that they consist of a phagocytically competent population. This is compatible with the conclusion of Wilton and Lehner that only a subpopulation of circulating neutrophils was phagocytosing normally,[9] perhaps because only fully competent cells emigrate from the blood to the tissues. A most pertinent fact is that patients with Behçet's syndrome do not appear to suffer unduly from infections, in marked contrast with states where there is clear dysfunction of individual neutrophils—for example, chronic granulomatous disease,[18] in which the patients are plagued by repeated episodes of infection, which may be fatal. It may be, therefore, that Behçet's syndrome is an example of a disease in which neutrophils behave normally as individuals but abnormally as a group.

Monocyte presence in the skin windows was frequently reduced. There are two reasons for not making too much of this observation. Firstly, there was no association with disease activity, and secondly, we have found monocyte accumulation to be aberrant in a wide variety of diseases. Nevertheless, monocytes may well play a part in the control of the acute cellular inflammatory response. If this is the case any decrease in their presence could lead to undue persistence of the neutrophil response. Only one of our 15 patients had a positive pathergy skin test, and this low prevalence of a positive test in British as compared with Turkish patients has been noted previously.[2] [19] Furthermore, the absence of an association between a positive test and leucocyte function abnormality argues against the importance of neutrophil hyperactivity in the pathergy reaction.

These results obtained in vivo in patients with Behçet's syndrome extend the in vitro observations of neutrophil hyperactivity previously reported. In vivo studies of leucocyte migration using such a skin window technique may be a useful aid to our understanding and monitoring of diseases characterised by an exaggerated cellular inflammatory response.

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