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Increased CD11/CD18 expression on peripheral blood leucocytes of patients with sarcoidosis

Z. SHAKOOR & A. S. HAMBLIN *Department of Immunology, United Medical and Dental Schools, St Thomas' Campus, London, UK*

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SUMMARY

Sarcoidosis is a multisystem disease of unknown etiology characterized by non-caseating granulomata, formed mainly from macrophages surrounded by lymphocytes and plasma cells. Using a novel method for the preparation of blood leucocytes for flow cytometry, we report increased expression of LeuCAMs (CD11/CD18) on peripheral blood leucocytes of 11 Caucasian and 10 Afro-Caribbean patients with sarcoidosis compared with age-, sex- and race-matched controls. Whilst the percentages of the cells expressing CD11/CD18 were no different, the density, expressed as mean fluorescence intensity (MFI), was greater for all leucocytes in sarcoids than in normal individuals. The expression of intercellular adhesion molecule-1 (ICAM-1), a ligand for LFA-1 which is expressed on all leucocytes, was not significantly different from normal, whereas HLA-DR was expressed more intensely on sarcoid monocytes ($P < 0{\cdot}01$) and blood lymphocytes ($P < 0{\cdot}005$) than control cells. Our findings are consistent with leucocyte activation although we were unable to confirm reports of elevated tumour necrosis factor-alpha (TNF-α) in the patients' plasma using an ELISA. Increased expression of adhesion molecules on peripheral blood leucocytes may play a role in the cellular extravasation, aggregation, and granuloma formation seen in sarcoidosis.

Keywords CD11/CD18 sarcoidosis leucocytes LeuCAM tumour necrosis factor

INTRODUCTION

Many immunological abnormalities accompany the formation of granulomata in sarcoidosis. Anergy in response to recall antigens in skin tests for delayed type-hypersensitivity [1], impairment of *in vitro* proliferative responses of blood mononuclear cells to antigens and mitogens [2], and peripheral blood lymphocytopenia [3] are often seen together with infiltration of affected tissues with lymphocytes, particularly CD4 lymphocytes, and monocytes [4–6]. Before their migration to sites of active disease [7,8] sarcoid mononuclear cells must adhere to the endothelium of capillaries. Once within tissues, sarcoid lymphocytes and monocytes have been shown to associate closely [9,10], an interaction which may favour both granuloma formation [11] and the enhanced lymphoproliferation which has been reported by cells retrieved from tissues such as the lung [10,12].

The LeuCAM leucocyte adhesion molecules (CD11a/CD18, CD11b/CD18 and CD11c/CD18) play important roles in mediating a wide variety of cell-substrate and cell-cell adhesion-related functions by leucocytes [13]. Using blocking MoAbs, it has been shown that LeuCAMs mediate the adhesion of leucocytes to endothelium [7] and the association of monocytes with lymphocytes which leads to lymphoproliferation [14]. Cells from patients with LeuCAM deficiency are incapable of performing adhesion-related functions, including emigration into inflammatory tissue lesions [15].

The role of LeuCAMs in adhesive processes is moderated by both quantitative and qualitative changes in their expression [13,16,17]. Monocytes and granulocytes possess internal latent pools of CD11b/CD18 and CD11c/CD18 which are mobilized to the plasma membrane in response to stimulation by mediators such as the complement fragment C5a, tumour necrosis factor-alpha (TNF-α) and phorbol esters [18]. Quantitative increases in adhesion molecule expression are associated with increased cell adhesion and migration both *in vitro* and *in vivo* [13,16].

The primary objective of this work was to quantify the expression of LeuCAM adhesion proteins on the peripheral blood leucocytes of patients with sarcoidosis using a method for preparing leucocytes for flow cytometry which we have recently described [19]. As a result of finding increased expression of LeuCAMs, a second objective was to study TNF-α levels in the plasma of the same patients. TNF-α, a cytokine produced principally by mononuclear phagocytes, has a wide spectrum of activity including the capacity to up-regulate LeuCAM expression [18,20] and has been reported to be elevated in the sera of patients with sarcoidosis [21].

Correspondence: Dr A. S. Hamblin, Department of Immunology, South Wing, St Thomas' Hospital, London SE1 7EH, UK.

PATIENTS AND METHODS

Study population

The diagnosis of sarcoidosis was established clinically and by biopsy in 21 patients (nine males and 12 females). Ten were Afro-Caribbean, mean age 36 years (range 21–63) and 11 were Caucasian, mean age 36 years (range 27–54). They were compared with age-, sex- and race-matched normal healthy donors: 10 Afro-Caribbeans, mean age 33 years (range 21–56) and 11 Caucasians, mean age 33 years (range 22–54). Patients were not receiving corticosteroids or other immunosuppressive therapy at the time of investigation or within the previous 12 months. Clinical information obtained for the patients included smoking habits, chest X-ray findings graded according to conventional criteria (Stage I, hilar adenopathy without parenchymal infiltrates; Stage II, hilar adenopathy with parenchymal infiltrates; and Stage III fibrosis or infiltrates without hilar adenopathy), symptom duration and organ involvement, serum angiotensin converting enzyme (SACE) levels and erythrocyte sedimentation rate (ESR) (Table 1), as well as lung function tests. Information was recorded for tests done within 1 week of blood being taken.

Preparation of leucocytes

Peripheral blood leucocytes were prepared for flow cytometry by a technique of rapid fix lysis [19]. Briefly, 1·0 ml of fresh heparinized venous blood was transported to the laboratory and added immediately to 1·0 ml of 0·4% formaldehyde (BDH, Poole, UK) and incubated for 4 min at 37°C in a 30 ml universal container (Bibby-Sterilin, Stone, UK). Twenty millilitres of warmed (37°C) 0·83% ammonium chloride (BDH), in 0·01 M Tris (Fisons, Loughborough, UK) lysing buffer pH 7·4 was then added and incubation continued until the erythrocytes were lysed (5–10 min). Leucocytes were sedimented by centrifugation at 160 ***g*** for 5 min at room temperature and the supernatant discarded. Cells were resuspended in 10 ml of PBS (Dulbecco's A, Oxoid, Basingstoke, UK) and centrifuged at 160 ***g*** for 5 min at room temperature. The supernatant was discarded and the pellet resuspended in 10 ml of PBS to repeat the wash. Finally, the cells were resuspended in 0·5 ml ice-cold RPMI 1640 (Gibco, Paisley, UK) containing 5% fetal calf serum (FCS, Gibco).

Monoclonal antibodies and cell staining

The mouse MoAbs used were MHM24 (CD11a) from Professor A. J. McMichael (Oxford, UK), 44 (CD11b), 3.9 (CD11c), and 52 (HLA-DR) from Dr Nancy Hogg (ICRF, London, UK), CD18 (Dakopatts, Denmark), UCHM1 (CD14) and UCHT1 (CD3) from Professor P. Beverley (University College and Middlesex School of Medicine, London, UK), RR1/1 (ICAM-1) from Dr R. Rothlein (Ridgefield, CT) and PDS1 (isotypic IgG1 control MoAb) from Dr S. Suleyman (United Medical and Dental Schools, London, UK). Control wells containing unlabelled cells and cells without MoAbs were run in parallel. Titration of antibodies before their use showed that the amounts used were saturating. The same batches of antibodies were used throughout. Twenty-five microlitres of each MoAb together with 25 μl of cell suspension were added to wells of flexible microtitre plate (Falcon, 3911), the contents were mixed by gentle tapping and incubated on ice for 30 min. The cells were centrifuged at 160 ***g*** for 5 min at 4°C and washed twice with 100 μl of 5% FCS/RPMI. Twenty-five microlitres of 1:50 dilution (in RPMI) of fluorescein-conjugated, rabbit anti-mouse $F(ab)_2$

Table 1. Clinical details of patients with sarcoidosis

Patient no.	Age (years)	Ethnic group/sex	Symptom duration (weeks)	Chest radiographs	Extrathoracic disease	SACE (U/ml)	ESR
1	27	AC/F	10	I	–	206	35
2	54	AC/F	300	I	–	243	100
3	26	AC/F	24	I	Parotid gland	156	58
4	56	AC/F	456	I	Skin	76	25
5	31	AC/M	5	I	–	156	14
6	25	AC/M	24	I	Uveitis	76	25
7	21	AC/M	4	II	Lymph node	184	42
8	31	AC/F	2	II	–	164	ND
9	30	AC/M	20	II	–	41	ND
10	63	AC/F	166	III	–	ND	68
11	32	C/M	4	0	Parotid gland	98	3
12	31	C/F	2	I	EN	ND	ND
13	30	C/M	20	I	–	ND	80
14	29	C/M	4	I	–	84	48
15	39	C/M	4	I	EN	63	25
16	27	C/F	24	I	–	ND	68
17	33	C/M	28	I	–	54	ND
18	33	C/F	160	II	–	116	20
19	53	C/F	21	III	–	ND	28
20	37	C/F	210	III	–	113	17
21	54	C/F	25	III	–	158	10

AC = Afro-Caribbean; C = Caucasian; M = male; F = female; – = none; SACE, serum angiotensin converting enzyme; ESR, erythrocyte sedimentation rate; EN, erythema nodosum; ND, not done.

(Dakopatts, Denmark) were then added to each well and incubated at 4°C in the dark for 30 min, followed by centrifugation at 160 ***g*** for 5 min at 4°C. The supernatant was discarded and the cells washed twice with 5% FCS/RPMI. The cells were resuspended in 300 μl of 1% paraformaldehyde (BDH) in PBS and transferred to FACScan tubes (Falcon, 2054) left at 4°C overnight in dark before flow cytometry using a FACScan (Becton Dickinson, Mountain View, CA).

Flow cytometry

Analysis was performed using Consort-30 software (Becton Dickinson) as previously described [19]. A minimum of 10 000 events was collected for each sample. Alignment of the detectors and the compensation were identical for all specimens. Cells were gated according to their forward scatter and side scatter into three populations representing the lymphocytes, monocytes and polymorphs. The purity of lymphocytes and monocytes was verified using CD3 and CD14 antibodies [19]. Positive cell staining was that seen on cells compared with a subclass-matched antibody of irrelevant specificity. For each population of cells results were expressed as the percentage of fluorescent cells and the mean fluorescence intensity (MFI) in arbitrary units transformed to a linear scale from the $\log_{10}$ channel number of mean fluorescence.

To examine whether differences seen were due to differences in the size of cells the forward scatter measurements on the different cell populations of the patients were compared with those of the controls.

TNF assay

Plasma samples obtained from centrifugation of the heparinized blood from all of the patients and controls were stored at −20°C before analysis of TNF-α levels in a single batch. Quantitative measurement of plasma TNF-α levels was done by a commercial sandwich enzyme immunoassay test kit (Biokine TNF test kit, T Cell Sciences, Cambridge, CT) and absorbance measured at 490 nm.

Statistical analysis

Data are expressed as means ± s.d. Comparisons were made by using a paired (for comparison of patients with controls) or unpaired (for a comparison of different ethnic groups) Student's *t*-test. Correlations were sought using Spearman's rank test.

RESULTS

CD11/CD18 expression

CD11/CD18 expression on sarcoid monocytes was higher than that of the controls for both ethnic groups (Fig. 1a, b). CD18 expression was also significantly increased on polymorphs (Fig. 1c, d) and lymphocytes (Fig. 1e, f) although differences in CD11 expression did not always achieve statistical significance. It is noteworthy that Afro-Caribbean controls expressed more CD11/CD18 than the Caucasian controls; significantly higher MFIs were found for CD18 on monocytes and polymorphs ($P<0{\cdot}01$ for all comparisons). In accordance with previous findings the proportions (%) of the different leucocyte populations expressing CD11/CD18 varied [19]. There were, however, no significant differences in the percentages of cells expressing the molecules when patients and controls were compared (Table 2).

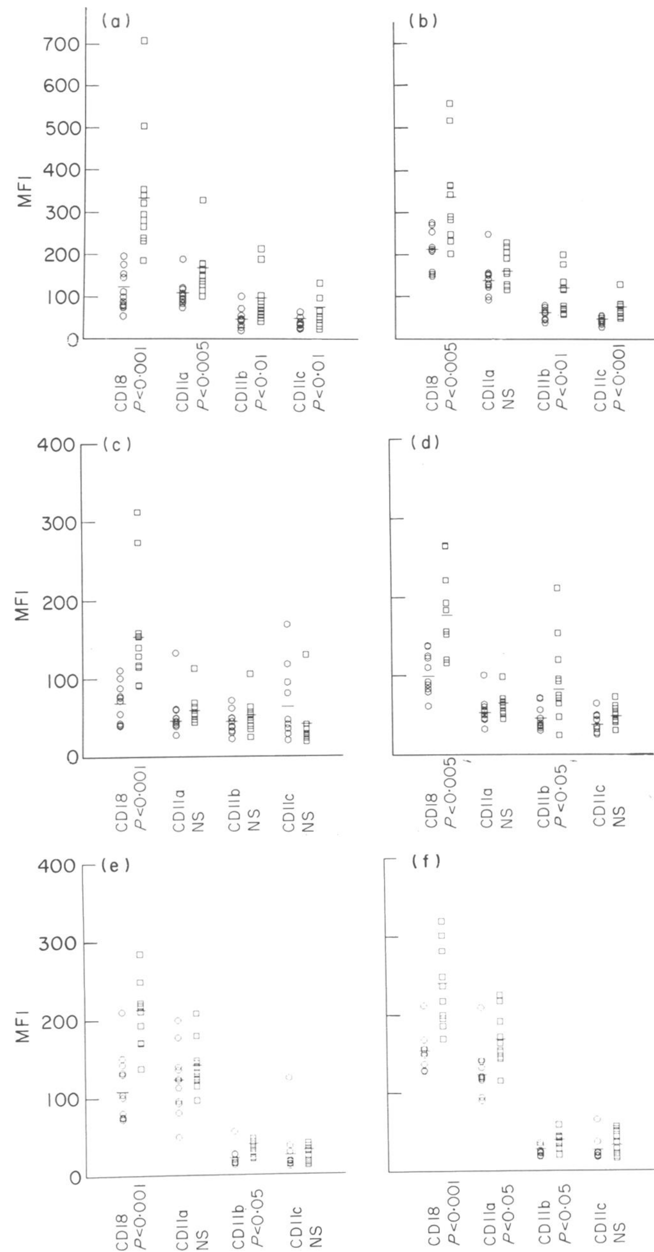

Fig. 1. Mean fluorescence intensity (MFI) of CD18, CD11a, CD11b and CD11c expression on the monocytes ((a) and (b)), polymorphonuclear leucocytes ((c) and (d)) and lymphocytes ((e) and (f)) of normal donors (○) and sarcoid patients (□). (a), (c) and (e) represent the results for Caucasian donors and (b), (d) and (f) for Afro-Caribbean donors. Each dot or square represents the results from one donor. The horizontal bars represent the mean values. Statistical significance is by the paired Student's *t*-test. NS, Not significant.

There were no significant differences in the mean forward scatters for monocytes (controls 217 ± 12; sarcoids 215 ± 11), polymorphs (controls 164 ± 31; sarcoids 170 ± 20) and lymphocytes (controls 136 ± 8; sarcoids 141 ± 8) showing that the differences in the MFIs were not attributable to differences in size.

Table 2. Percentages of peripheral blood leucocytes expressing CD11b/CD18 molecules

	Normals		Sarcoids	
	AC	C	AC	C
Monocytes				
CD18	99±0·34	99±0·78	99±0·44	99±0·49
CD11a	99±0·35	99±0·96	99±0·44	99±0·27
CD11b	95±1·96	79±16·33	97±3·47	97±0·68
CD11c	93±1·45	65±27·99	96±5·98	94±7·07
Polymorphs				
CD18	99±0·14	98±1·27	99±0·33	99±0·45
CD11a	99±0·27	98±2·21	99±0·11	97±8·08
CD11b	97±3·11	66±29·64	99±1·18	97±6·17
CD11c	75±22·06	50±27·12	84±18·22	79±25·66
Lymphocytes				
CD18	99±0·36	98±2·97	99±0·41	99±0·71
CD11a	99±0·36	97±4·40	99±0·41	99±2·28
CD11b	32±14·20	30±11·60	36±17·70	40±17·05
CD11c	20±5·72	22±11·96	25±16·86	24±12·24

AC, Afro-caribbean; C, Caucasian. All values are mean±s.d.

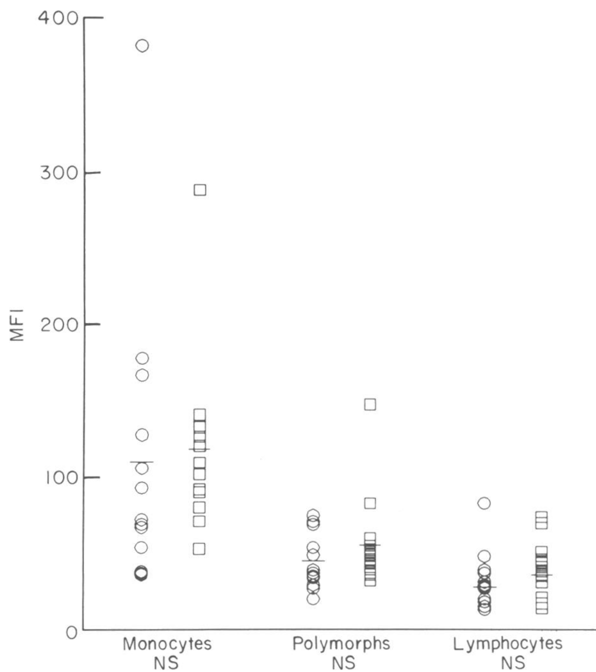

Fig. 2. Mean fluorescence intensity (MFI) of ICAM-1 expression on monocytes, polymorphonuclear leucocytes and lymphocytes in normal donors (○) and sarcoid patients (□). Results are presented for 13 normal and 13 sarcoid donors each comprising three Caucasians and 10 Afro-Caribbeans. The horizontal bars represent the mean values. Statistical significance is by the paired Student's *t*-test. NS, Not significant.

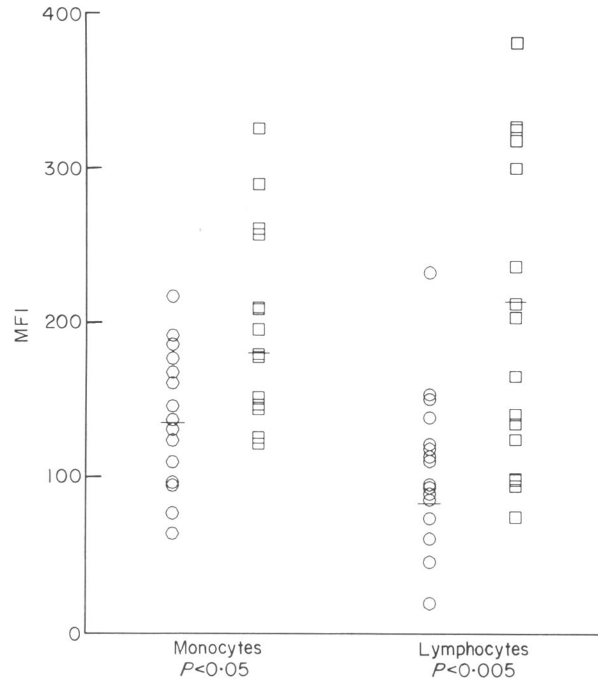

Fig. 3. Mean fluorescence intensity (MFI) of HLA-DR expression on monocytes and lymphocytes in normal donors (○) and sarcoid patients (□). Results are presented for 19 normal and 19 sarcoid donors, each comprising nine Caucasians and 10 Afro-Caribbeans. The horizontal bars represent the mean values. Statistical significance is by the paired Student's *t*-test.

ICAM-1 expression

MFIs for ICAM-1 were not significantly different between patients and controls for any leucocytes (Fig. 2). In addition, there was no difference in the percentage of cells expressing ICAM-1 which was found on the majority of monocytes (99±0·61%), polymorphs (98±1·78%) and lymphocytes (95±4·99%) in accordance with previous studies [22].

HLA-DR expression

MFIs for HLA-DR on both monocytes and lymphocytes were higher in sarcoids than in controls (Fig. 3). A higher percentage of sarcoid lymphocytes (42±13%) expressed HLA-DR compared with controls (23±7%) whereas the proportions of positive monocytes were no different (normal mean, 95±5·5%, sarcoid mean 97±4·2%). HLA-DR was not expressed on blood polymorphs.

TNF assay

Plasma samples screened for the presence of free TNF-α failed to show presence of the cytokine in patients or controls from either ethnic group. All samples were below 15 pg/ml (upper limit of normal range 67 pg/ml).

Clinical correlations

Significant correlations ($P<0·05$) were found for monocyte CD11b ($r=0·554$), CD11c ($r=0·459$), and polymorph CD11b ($r=0·628$) expression and SACE levels. However, no correlation was found between CD11/CD18 levels and ESR or duration of disease. HLA-DR expression correlated with CD18 ($r=0·476$), CD11b ($r=0·520$), and CD11c ($r=0·45$) only on monocytes. The only significant correlation for lung function

tests was between a fall in SVC (slow vital capacity) and monocyte CD11a ($r = -0{\cdot}722$) and CD11b ($r = -0{\cdot}588$) and neutrophil CD11a ($r = -0{\cdot}689$) and CD11b ($r = -0{\cdot}644$). Thus, higher levels of CD11 were associated with lower SVCs. No correlation was seen with either transfer factor or transfer coefficient.

DISCUSSION

CD11/CD18 molecules play a role both in leucocyte adherence and migration into tissues and in interleucocyte adherence. Since there is mononuclear cell infiltration into affected tissues and abnormal leucocyte function in sarcoidosis, the abnormal expression we have seen here may play a significant role in the disease process.

The exact role increased levels of CD11/CD18 play in increased leucocyte adherence are unclear. Increased expression of LeuCAMs on neutrophils has been observed in patients undergoing haemodialysis [16], suffering from burns [23], and systemic lupus erythematosus [24]. The neutropenia and microvascular injury associated with these conditions may thus arise from enhanced neutrophil adhesion to vascular endothelium promoted by the increased CD11/CD18 expression. Quantitative changes in expression have also been associated with increased adherence *in vitro* [25]. Because several CD11/CD18-mediated functions occur without an increase in their expression on the cell surface [17] it is believed that conformational changes are equally if not more important than quantitative changes [13]. Recently it has been suggested that rolling of neutrophils on vascular endothelial cells (an early event in inflammation) mediated by selectins precedes adhesion strengthening through CD11/CD18. This strengthening is promoted by changes in LeuCAM avidity following activation of cells by chemoattractants [26]. From this we conclude that increased expression may predispose cells to increased adherence when activation occurs in the environment of an inflammatory stimulus.

Differences between patients and controls found in our study may reflect genetic differences, the relative immaturity of patients' cells or their activation by cytokines or unknown factors. In terms of genetic control of expression, patients with Trisomy 21, Down's syndrome, are known to express increased CD18 due to the duplication of chromosome 21 on which the gene is located [27]. This increased expression may be responsible for some of the immunological abnormalities reported in Down's patients [28]. Apart from these unusual circumstances nothing is known about the genetic regulation which may lead to differences in the levels of CD11/CD18 expression. That genetic elements may play a role is perhaps supported here by the observation that both normal and sarcoid Afro-Carribean mean levels were higher than Caucasian normal and sarcoid mean levels. However, it is equally possible that environmental factors may differently influence the two ethnic populations.

The second possibility is that the rate of migration of cells from the bone-marrow through the blood to tissues is faster in sarcoid patients than in normal individuals, resulting in the accumulation of less mature cells in the circulation. Increased numbers of T lymphocytes, particularly $CD4^+$ lymphocytes, as well as alveolar macrophages, which are morphologically and antigenically similar to monocytes, are found in the affected lungs of patients with sarcoidosis, providing evidence for the recent and abnormal migration of lymphocytes and monocytes into the lungs [4,5,29]. Differences in expression of CD11/CD18 are known to occur during leucocyte differentiation [30] and elevated levels may therefore reflect a different maturational level of cells in patients compared with controls. The fact that polymorphs, which are not usually seen in substantially increased numbers in tissue infiltrates in sarcoidosis, also expressed more CD11/CD18 suggests that this is not the explanation. Furthermore, no evidence has been found for increased leucocyte adhesion molecule expression in other inflammatory diseases such as rheumatoid arthritis [31], inflammatory bowel disease [32] and systemic vasculitis (Ahmad, personnel communication) where there is also leucocyte infiltration into tissues, and evidence of increased recruitment from bone-marrow.

Increased LeuCAM expression on sarcoid peripheral blood leucocytes may be due to cellular activation. This may be direct activation by an as yet unidentified infectious agent acting at the cell surface or within the cell. Viruses have been reported to cause increased CD11/CD18 expression on leucocytes [33]. Alternatively, cytokines released into the blood stream from the sites of disease may be responsible. Lymph node extracts of patients with active sarcoidosis have been shown to contain significant levels of interferon-gamma (IFN-γ), TNF-α and IL-1β, and lymph node IFN-γ levels have been shown to correlate with the serum IFN-γ levels [34]. Increased plasma levels of TNF-α have also been reported in patients with sarcoidosis [21]. However, plasma levels of TNF-α in the patients in the present study were not elevated, suggesting it is not systemically involved in the increased expression. A number of cytokines other than TNF-α and IFN-γ can rapidly increase the expression of LeuCAMs *in vitro* on peripheral blood phagocytes but not lymphocytes [35,36] and are thus candidates for causing the elevated expression on monocytes and polymorphs reported here.

Further support for leucocyte activation comes from our finding that HLA-DR expression was also elevated on both lymphocytes and monocytes. HLA-DR expression on sarcoid monocytes correlated well with LeuCAM expression and may be due to cytokines as outlined above. The increased proportion of lymphocytes expressing HLA-DR could be due to activation or to differences in the composition of the lymphocyte populations in the two groups. A higher proportion of B lymphocytes is found in sarcoid peripheral blood as a result of redistribution of T lymphocytes to sites of disease activity [37]. No attempt was made here to look at CD11/CD18 expression on different lymphocyte populations, the composition of which may vary between the patients and the controls.

Differences in the composition of sarcoid peripheral lymphocytes may also underlie the increased level of expression of CD11/CD18 seen on these cells. In addition to the contribution from altered proportions of T and B lymphocytes, it has been reported that subpopulations of T cells also express different levels of CD11/CD18 [38] and that conversion of T cells from naive to memory type increases the level of expression of the adhesion molecules [39]. Such alterations in subpopulations have recently been reported to affect CD11a/CD18 expression on Down's syndrome lymphocytes [40]. Whether differences in the composition of lymphocyte populations result in an apparent overall increase in lymphocyte CD11/CD18 in sarcoidosis is currently under investigation.

Measurement of disease activity in sarcoidosis is difficult and

usually relies on the examination of a number of markers such as SACE, ESR, X-ray stage and lung function tests. Although we did not find correlations between disease duration, ESR, chest X-ray stage and most lung function tests in this small group of patients, our finding that elevated levels of SACE correlated with CD11/CD18 expression on monocytes and neutrophils suggests that CD11/CD18 expression is worthy of investigation as a marker for disease activity in systemic lupus erythematosus [24]. The reason for the negative correlation with SVC, but not with any other lung function tests, is not clear. Although not a very good marker of disease activity, SVC decreases in chronic sarcoid and thus a negative correlation is further suggestive evidence that CD11/CD18 expression may be of interest as a marker of more persistant disease. A better understanding of the relationship of CD11/CD18 expression to disease activity requires investigation of a larger group of patients during the natural development and the treatment of their disease.

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