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Increased CD11/CD18 expression on the peripheral blood leucocytes of patients with HIV disease: relationship to disease severity

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SUMMARY

In HIV disease increased adhesion between leucocytes themselves and between leucocytes and endothelium may contribute to cell loss and viral spread. Using a novel method for the preparation of blood leucocytes for flow cytometry, we report increased expression of leucocyte adhesion molecules (LeuCAMs) (CD11/CD18) on peripheral blood leucocytes of patients with HIV disease compared with normal controls. Patients were divided into two groups on the basis of CD4 T lymphocyte numbers (those with $>0{\cdot}5\times10^9/l$ and those with $<0{\cdot}2\times10^9/l$), and assessed for p24 antigen expression, viral load and serum tumour necrosis factor (TNF) levels as well as LeuCAM expression. Patients with $<0{\cdot}2\times10^9/l$ CD4 cells had more p24 antigen and more HIV infectious virus and more serum TNF than those with $>0{\cdot}5\times10^9/l$. Whilst the percentages of only monocytes and polymorphs expressing CD11b were significantly increased in patients with the least CD4 cells, the density of LeuCAMs, expressed as mean fluorescence intensity (MFI), was significantly increased on all leucocytes, with the most significant increases being seen on patients with the fewest CD4 T cells. Our findings are consistent with leucocyte activation by a soluble factor, although we could find no correlation between levels of TNF and LeuCAM expression. The increased expression of adhesion molecules on peripheral blood leucocytes could play a role in the cellular extravasation and aggregation seen in HIV disease.

Keywords HIV LeuCAMs CD11/CD18 tumour necrosis factor

INTRODUCTION

The leucocyte adhesion molecules (CD11a, b, c and CD18, or LeuCAMs) [1] mediate a wide range of adhesion-dependent functions, including adherence to endothelial cells [2,3]. A critical component of the host defence is the ability to enhance leucocyte adhesion in a controlled manner. This is achieved by quantitative and qualitative changes in the expression of CD11/CD18 molecules, and by the level of expression or generation of ligands [3]. Processes which increase the expression of the adhesion proteins may favour the increased adherence of cells to other cells and to endothelium. In HIV disease this may favour both viral spread and cell loss.

In HIV infection both $CD4^+$ T lymphocytes and monocytes/macrophages are infected with the virus [4,5]. Selective and progressive depletion of $CD4^+$ T lymphocytes, which is such a striking feature of this disease [6,7], occurs as a result of many pathological processes [8], including the formation of syncytia which are seen *in vivo* [9] as well as *in vitro* [10]. Antibodies to LeuCAMs have been shown to inhibit HIV-induced syncytium formation *in vitro* [11,12], suggesting that these molecules are important in the pathological process. Viral spread occurs when cells migrate from peripheral blood to tissues, a process which initially requires adherence to endothelial cells. Cells such as monocytes, in which infectious virions accumulate within cytoplasmic vacuoles without causing lysis [13], are thought to be particularly important for viral spread through the body when they migrate from blood into tissues and become macrophages.

LeuCAM adhesion molecule expression may be increased both by infection by the virus or activation of cells by soluble factors such as cytokines. HIV-1 infection of cell lines results in increased expression of CD11a/CD18 [14]. The cytokine tumour necrosis factor-alpha (TNF-α) has been shown *in vitro* to increase surface expression of LeuCAM molecules on monocytes and granulocytes [15–17]. Raised serum levels of TNF-α have been reported in HIV-1-infected patients [18], and substantially increased *in vitro* production of TNF-α by peripheral blood monocytes from HIV-1-infected individuals activated *in vitro* has been described [19].

The objective of this study was to investigate LeuCAM expression on peripheral blood lymphocytes, granulocytes and monocytes using a recently described method to prepare

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Table 1. Demographic features of patients

No.	Sex	Age	Race	CDC stage	Total lymphocytes $\times 10^9/l$	$CD4^+$ lymphocytes $\times 10^9/l$	$CD8^+$ lymphocytes $\times 10^9/l$	HIV-1 p24 antigen pg/ml	HIV-1 titre TCID/10^6 PBMC
Group 1									
1	M	23	Cau	II	1·7	0·68	0·51	<6·0	<0·5
2	M	24	Cau	II	4·1	1·03	2·62	<6·0	<0·5
3	M	33	Cau	II	4·3	0·56	3·35	<6·0	<0·5
4	M	34	Cau	II	1·6	0·62	0·69	<6·0	<0·5
5	M	28	AC	II	2·7	0·76	1·27	<6·0	<0·5
6	M	25	Cau	II	1·6	0·53	0·83	<6·0	<0·5
7	M	29	Cau	II	3·32	0·86	1·95	<6·0	<0·5
Group 2									
8	M	28	Cau	IVC	1·6	0·03	1·18	<6·0	5
9	M	42	Cau	IVA	0·6	0·01	0·38	53·0	50
10	M	39	Cau	II	0·42	0·05	0·24	200·0	NT
11	M	41	Cau	IVC	0·4	0·004	0·32	75·4	50
12	M	46	Cau	IVC	1·5	0·05	1·35	48·6	5
13	M	32	Cau	II	1·4	0·14	1·06	630·0	5
14	F	31	Cau	IVC	1·09	0·07	0·84	34·7	50

Cau, Caucasion; AC, Afro-Caribbean; PBMC, peripheral blood mononuclear cells; TCID, tissue culture infective dose; NT, not tested.

leucocytes, in which cell adhesion molecule up-regulation during cell manipulation is prevented [20]. Expression has been studied in relation to disease progression as determined by CD4 T cell numbers and viral load. In view of reports of elevated TNF-α in the serum of HIV-infected individuals and the role this plays in up-regulation of CD11/CD18 [15–18], the levels of TNF-α in the serum of patients and controls in the study were determined and correlated with LeuCAM expression.

PATIENTS AND METHODS

Study populations

Fourteen HIV-1^+ donors (13 males, 1 female) were recruited from patients attending the Department of Genito-Urinary Medicine, St. Thomas' Hospital, London, UK. The patients, who were not receiving any anti-HIV therapy at the time of the study, were classified into two groups according to their CD4 T lymphocyte count (Table 1). CD4 and CD8 lymphocyte numbers were determined from whole-blood staining with Simultest reagents (Becton Dickinson, Mountain View, CA) and analysed by flow cytometry (FACScan, Becton Dickinson). Group 1 (mean age 28 years) had a CD4 count of $>0·5\times10^9/l$ (mean $0·72\times10^9/l$). Group 2 (mean age 37 years) had a CD4 count of $<0·2\times10^9/l$ (mean $0·05\times10^9/l$). Table 1 shows the demographic features of the patients who were staged according to the Centre for Disease Control (CDC) criteria [21]; all Group 1 patients and two Group 2 patients had asymptomatic HIV disease (CDC stage II), one Group 2 patient had constitutional disease (CDC stage IVA), and the remaining four Group 2 patients had AIDS (CDC stage IVC). Patients' AIDS-defining illnesses were *Pneumocystis carinii* pneumonia (patient 8), cytomegalovirus retinitis (patient 11), candidiasis (patient 12) and *Toxoplasma* (patient 14); diagnosis had been made 4–6 months before this study. All but two of the patients were free of obvious infection at the time of blood being taken and had had no acute infectious episodes in the previous 3 months. Patient 5 was a hepatitis B carrier of low infectivity (hepatitis B surface antigen and e antibody-positive) and had *Haemophilus influenzae* isolated from sputum. Patient 14 had oral candida. A quantitative enzyme immunoassay (Abbott Diagnostics) was used to measure HIV-1 p24 antigen levels in serum taken at the same time as other laboratory studies. Titres of infectious HIV-1 in the peripheral blood mononuclear cells (PBMC) were determined as previously described [22]; infectivity titres were expressed as tissue culture infective dose (TCID)/10^6 PBMC.

Control donors were six healthy, HIV-1^- males, mean age 29 years (range 22–41 years). The control subjects had a mean CD4 T cell count of $0·79\times10^9/l$ (range $0·53–1·18\times10^9/l$).

Preparation of leucocytes

Peripheral blood leucocytes from patients and controls were prepared for MoAb labelling by the rapid fixation and lysis technique previously described [20]. Briefly, a sample of venous blood was collected into heparin at a final concentration of 20 U/ml (Leo Laboratories Ltd). A 0·5-ml aliquot of blood was immediately mixed with 0·5 ml of 0·4% formaldehyde/PBS (Dulbecco's A, Unipath) prewarmed to 37°C and incubated at 37°C for 4 min. An excess (20 ml) of lysing buffer pH 7·4 (0·83% ammonium chloride/0·01 M Tris) was added and incubation continued at 37°C until erythrocyte lysis occurred (approximately 4 min). The cells were centrifuged at 160 *g* for 5 min at room temperature, the supernatant was discarded and the leucocyte-rich pellet resuspended in RPMI tissue culture medium (Northumbria Biologicals Ltd) containing 5% heat-inactivated (56°C for 30 min) fetal calf serum (FCS; Gibco). The leucocytes were then washed twice in 5 ml of FCS/RPMI (160 *g*, room temperature, 5 min) and finally resuspended in 0·5 ml of FCS/RPMI and held on ice before MoAb labelling.

Monoclonal antibody staining

A 25-μl aliquot of the leucocyte suspension was mixed with an equal volume of each of the mouse MoAbs. The antibodies used

Table 2. Percentage of peripheral blood leucocytes expressing leucocyte adhesion molecules (LeuCAMs)

	Controls		Patient Group 1		Patient Group 2	
	Mean	(95% CI)	Mean	(95% CI)	Mean	(95% CI)
Monocytes						
CD11a	99·6	(99·5–99·8)	99·8	(99·7–99·9)	99·7	(99·6–99·9)
CD11b	59·0	(46·8–71·2)	61·0	(42·8–79·3)	86·8	(77·1–96·5)*
CD11c	26·5	(19·6–33·4)	43·3	(21·3–65·3)	50·3	(33·9–66·6)*
CD18	99·6	(99·6–99·7)	99·7	(99·4–99·9)	99·7	(99·4–99·9)
Polymorphs						
CD11a	98·4	(97·1–99·6)	98·8	(99·7–99·9)	98·5	(97·5–99·5)
CD11b	31·9	(15·5–48·1)	47·3	(22·9–71·6)	87·0	(75·8–98·2)**
CD11c	4·5	(1·1–7·9)	6·1	(1·0–11·3)	6·6	(4·4–8·8)
CD18	98·8	(97·8–99·9)	99·8	(99·6–99·9)	99·7	(99·5–99·9)
Lymphocytes						
CD11a	98·0	(96·7–99·2)	99·3	(98·7–99·9)	98·0	(96·3–99·6)
CD11b	15·4	(9·7–21·1)	11·0	(4·5–17·6)	16·9	(8·5–25·3)
CD11c	3·2	(2·1–4·4)	7·3	(3·7–10·9)	9·2	(4·3–14·1)
CD18	98·0	(96·1–99·3)	99·3	(98·4–100.0)	97·9	(96·6–99·3)

* $P < 0{\cdot}05$ compared with controls.
** $P < 0{\cdot}005$ compared with both controls and Group 1 patients.
CI, Confidence interval.

were UCHT1 (CD3, [23]), UCHM1 (CD14, [24]), MHM24 (CD11a, [25]), 44 (CD11b, [26]), 3.9 (CD11c, [27]), MHM23 (CD18, [25]). All the MoAbs were IgG1 except UCHM1, which was IgG2a. The mixtures were incubated on ice for 30 min, washed twice with FCS/RPMI, and then incubated with a 1 : 50 dilution of FITC-conjugated rabbit anti-mouse Fab_2 (Dakopatts, Glostrup, Denmark). Control cells were incubated without the mouse MoAbs. The cells were washed twice as above and resuspended in 300 μl of 1% paraformaldehyde (Agar Aids Ltd, Stanstead, UK) in PBS and stored at 4°C, in the dark, for 24 h before analysis.

Flow cytometry
The stained cells were analysed using Consort 30 program on a FACScan flow cytometer (Becton Dickinson). Ten thousand events were accumulated from each stained leucocyte population. Alignment of the detectors and the compensation were identical for all specimens. Using forward scatter/side scatter (FSC/SSC) dot-plot profiles, distinct monocyte, lymphocyte and polymorph populations were identified and gated. The percentage of labelled cells was determined for each gated population. Cells were analysed for the density of fluorescence expressed as the mean fluorescence intensity (MFI) in arbitrary units transformed to a linear scale from the $\log_{10}$ channel number of mean fluorescence.

TNF-α assay
Serum levels of TNF-α were determined on samples from all patients and controls by enzyme-immunoassay (Medgenix SA, Fleurus, Belgium). Sample or standard (200 μl) was incubated in a microtitre plate coated with MoAbs directed against TNF-α. After incubation, any unbound material was removed by washing, and horseradish peroxidase-conjugated anti-TNF-α serum added to the plate. Unbound material was removed by washing, tetramethyl-benzidine solution containing peroxide was added, and a blue colour developed. The enzyme reaction was stopped by the addition of 1 M sulphuric acid and the intensity of the colour read on a spectrophotometer at 450 nm with a reference filter at 630 nm. The concentration of TNF-α in the samples was calculated from the standard calibration curve and expressed in pg/ml.

Statistical analysis
Statistical comparison of flow cytometry data was carried out using unpaired Student's *t*-test. TNF-α and HIV-1 p24 levels and HIV infectious virus titres (TCID/10^6 PBMC), were compared using the Mann–Whitney test. Correlations between LeuCAM expression and virological and immunological parameters were sought using Spearman's rank coefficient or multiple linear regression.

RESULTS

LeuCAM expression

The results obtained for the normal donors both in terms of the percentage of stained cells and levels of LeuCAM expressed were the same as those obtained in previous studies [28–30].

There was no difference between patients and controls in the percentage of monocytes expressing CD11a or CD18. A significant increase was seen in the percentage of monocytes from Group 2 patients expressing CD11b and CD11c compared with controls (Table 2). Group 2 patients' monocytes had a significantly increased density of expression of all four LeuCAM molecules compared with the controls (Fig. 1).

No differences were seen between patients and controls in the percentage of polymorphs expressing CD11a, CD11c and CD18. Group 2 patients showed a significantly increased

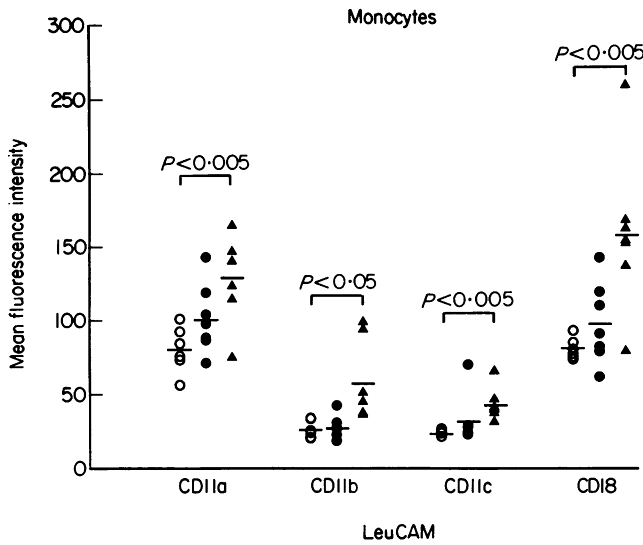

Fig. 1. Mean fluorescence intensity of CD11a, CD11b, CD11c and CD18 expression on monocytes from controls (○), Group 1 patients (●), and Group 2 patients (▲). Each symbol represents the results from one donor. The horizontal bars represent the mean values. Statistical significance is by the unpaired Student's *t*-test. LeuCAM, Leucocyte adhesion molecules.

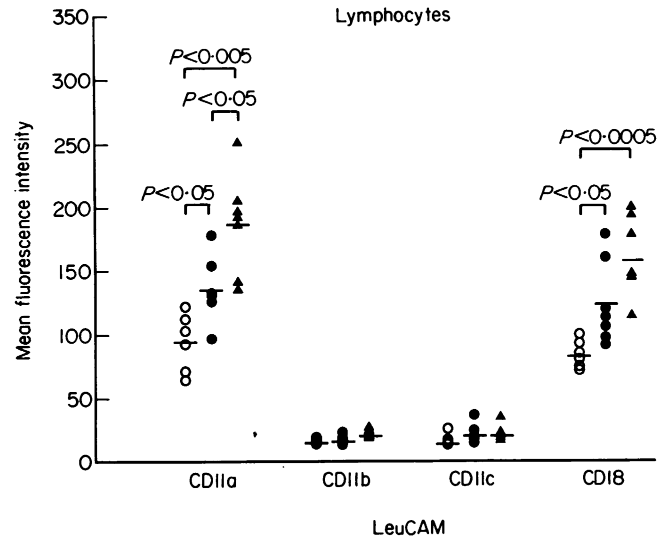

Fig. 3. Mean fluorescence intensity of CD11a, CD11b, CD11c and CD18 expression on lymphocytes from controls (○), Group 1 patients (●), and Group 2 patients (▲). Each symbol represents the results from one donor. The horizontal bars represent the mean values. Statistical significance is by the unpaired Student's *t*-test. LeuCAM, Leucocyte adhesion molecules.

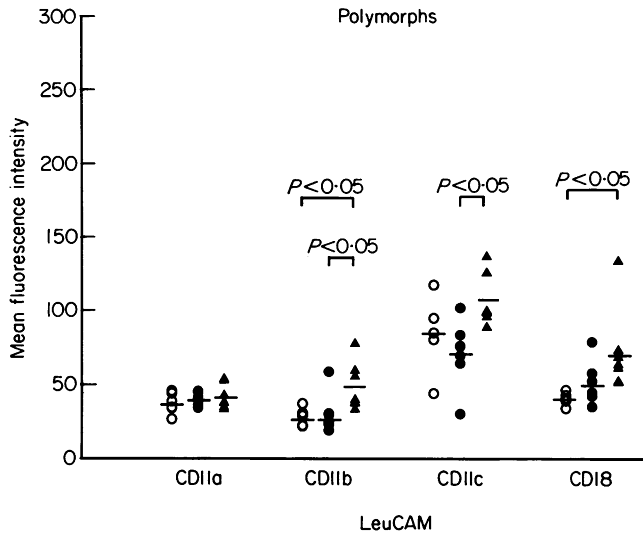

Fig. 2. Mean fluorescence intensity of CD11a, CD11b, CD11c and CD18 expression on polymorphs from controls (○), Group 1 patients (●), and Group 2 patients (▲). Each symbol represents the results from one donor. The horizontal bars represent the mean values. Statistical significance is by the unpaired Student's *t*-test. LeuCAM, Leucocyte adhesion molecules.

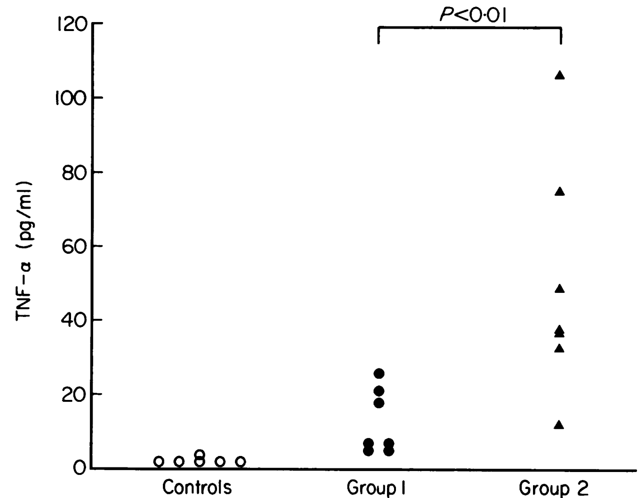

Fig. 4. Serum TNF-α levels on controls (○), Group 1 patients (●), and Group 2 (▲) patients. Each symbol represents the results from one donor. Statistical significance is by the Mann–Whitney test.

percentage of cells expressing CD11b compared with both controls and Group 1 patients (Table 2). Compared with controls, polymorphs from Group 2 patients showed a significant increase in density of expression of CD11b and CD18 (Fig. 2). Furthermore, cells from Group 2 patients compared with Group 1 patients had a significant increase in density of both CD11b and CD11c.

There was no difference between patients and controls in the percentage of lymphocytes expressing CD11a, b, c or CD18 (Table 2). Lymphocytes from both groups of patients showed a significant increase in the density of CD11a and CD18 molecules compared with controls (Fig. 3). Furthermore, Group 1 and Group 2 patients differed significantly in their expression of CD11a.

Levels of TNF-α

Figure 4 shows the TNF-α levels in patients and controls. TNF-α was undetectable (i.e. <3 pg/ml) in five of the six control

subjects. Six of the seven Group 1 patients had TNF-α levels within the normal assay range (3–20 pg/ml). Six of the seven Group 2 patients had elevated levels of TNF-α which were significantly higher than those in Group 1 patients (the difference between the medians being 30·9, with 95% confidence interval from 10·8 to 69·7; $<P=0{\cdot}01$).

Patients in Group 1 and Group 2 differed significantly in their amounts of HIV infectious virus (TCID/10^6 PBMC), their CD4 lymphocyte count ($P<0{\cdot}005$) and their HIV-1 p24 antigen levels ($P<0{\cdot}05$). No correlation was found between LeuCAM adhesion protein expression on any leucocyte and TNF-α levels, CD4 lymphocyte numbers, p24 antigen levels, and HIV-1 titre (TCID/10^6 PBMC).

DISCUSSION

This study shows that there is increased expression of LeuCAMs on peripheral blood monocytes, polymorphs and lymphocytes in patients with HIV-1 infection. Increases in LeuCAM expression have been seen in our studies using the same technique in patients with sarcoidosis [28], but not in patients with rheumatoid arthritis [29] and inflammatory bowel disease [30]. It is therefore interesting to speculate whether similar immunopathological mechanisms are responsible for increased LeuCAM expression seen in HIV disease and sarcoidosis, but are absent from rheumatoid arthritis and inflammatory bowel disease.

The increase in LeuCAM expression was greatest on cells in patients with a CD4 T cell count of $<0{\cdot}2\times10^9/l$. Since CD4 T cell count has been shown to be the most reliable laboratory marker for monitoring progression of HIV disease in patients [31], this suggests increased expression with disease progression. Furthermore, Group 2 patients generally had more severe disease than Group 1 patients by CDC staging, had higher levels of circulating p24 antigen, and proved easier to isolate virus from (Table 1). Thus patients with more advanced disease show increased LeuCAM expression. However, there was no formal correlation between levels of LeuCAM expression and these markers of disease progression/severity.

The increase in the proportion of monocytes and polymorphs expressing CD11b (Table 2) and the increased level of expression of this molecule and of CD18 are consistent with activation of these cells in the circulation. Davidson *et al.* [32] have reported increased CD11b on monocytes from HIV-infected individuals with ARC or AIDS, and Desroches & Rigal [33] have reported increased CD11a and CD18 on HIV-infected monocytes in ARC and AIDS patients, and concluded that viral infection itself or cell activation via a soluble mediator were responsible. Increase in expression of LeuCAMs has been demonstrated following *in vitro* infection of monocyte cell lines [14,34]. The finding here that polymorphs, which are not known to be infected with the virus, show similar changes to monocytes suggests that activation via some extrinsic mechanism is at least in part responsible. It is conceivable that infectious agents other than HIV could cause similar changes in LeuCAM expression. Most of our patients were free from obvious infection at the time of blood being taken and in the previous 3 months. However, we cannot rule out the presence of undetected infections, and that these could be responsible for the changes seen.

A variety of different cytokines have been shown to increase CD11/CD18 expression on polymorphs and monocytes [16,17]. Levels of TNF-α have been shown to be raised in HIV-1-infected patients [18] and the cytokine has been shown to increase expression of CD11b/CD18 and CD11c/CD18 on monocytes and granulocytes following *in vitro* stimulation of leucocytes [15]. We have shown here in agreement with Lahdevirta *et al.* [18] that patients with more advanced HIV-1 disease have significantly elevated serum levels of TNF-α as detected by immunoassay. However, there was no correlation in our study between TNF-α levels and density of LeuCAM expression, suggesting either that the detected TNF-α was not biologically active or that another cytokine or activation factor was responsible for the changes seen.

We have shown an increase in density of CD11a and CD18 on the lymphocytes from both groups of HIV-1-infected patients. An increase in level of expression of CD11a and CD18 has also been described in the CD3, CD8 and CD16 lymphocyte populations of HIV^+ individuals [33]. Whilst HIV infection itself may lead to increased expression on lymphocytes as shown for the T lymphocyte cell lines (H9) [14] and CEM [35], differences in the composition of peripheral lymphocytes in the patients may influence the increased expression seen. Patients have a decrease in CD4 T cells and an increase in the CD8 population, which results in a reduction of the CD4/CD8 ratio. A positive correlation between CD11a expression and inversion of CD4/CD8 ratio together with an increase in the $CD8^+$ $CD57^+$ subset has been reported in patients with Down's syndrome [36]. Further investigation is required to see if increased expression is associated with a particular lymphocyte subset.

The exact role that increased levels of LeuCAMs play in altering adherence is not known. Because certain CD11/CD18-mediated functions occur without an increase in the level of their expression [37] it is believed that conformational changes are equally if not more important [2]. Increased expression may however predispose cells to show increased adherence to other cells in the environment of an inflammatory stimulus. In this way increased LeuCAM expression on leucocytes from HIV-1-infected individuals may lead to viral spread via promotion of cell-to-cell contact. Our study suggests that this predisposition may become more pronounced as the disease progresses.

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