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L-selectin in patients with common variable immunodeficiency (CVID): a comparative study with normal individuals

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SUMMARY

L-selectin is one of the key members of the selectin family of adhesion molecules and initiates leucocyte attachment to specialized high endothelial venules. The shed form, which retains functional activity, can be detected in biological fluids and is increased in diseases of many kinds. In the present study, we investigated L-selectin expression on leucocytes and measured the soluble form in the plasma of healthy individuals and patients with CVID. A significant loss of L-selectin expression is found on CVID B cells, which is marked by the presence of a substantial population of L-selectin-negative B cells in the peripheral blood of some CVID patients. On $CD4^+$ T cells, the loss in L-selectin expression affects mostly the $CD45RO^+$ population. Peripheral blood leucocytes other than lymphocytes express L-selectin molecule normally. Moreover, soluble L-selectin was detected in significantly increased levels in CVID plasma compared with healthy controls. Our data suggest that the loss of L-selectin expressed by lymphocytes may be due to increased or aberrant lymphocyte activation in CVID patients who remain immunodeficient, and down-regulation of L-selectin from these lymphocytes may significantly contribute to the elevated levels of soluble L-selectin in the plasma, which may in turn affect further lymphocyte trafficking.

Keywords common variable immunodeficiency L-selectin B cells T cells

INTRODUCTION

CVID is a human primary immunodeficiency characterized by defective antibody production *in vivo*. Thus patients suffer from recurrent sinopulmonary infections, often associated with chronic lung disease [1]. The etiology is still unknown, but cellular abnormalities of various kinds including abnormal B cells phenotypes and functions as well as defects in T cells and accessory cells have been reported [2]. The heterogeneity of the disease in clinical and immunological terms makes it difficult to identify a clear and consistent pattern of abnormalities. In order to overcome this difficulty attempts have been made by many researchers to divide CVID patients into subgroups based on CVID lymphocyte responses to stimulations, such as *Staphylococcus aureus* Cowan (SAC) or SAC + IL-2 [3], SAC + pokeweed mitogen (PWM) [4]. A method has also been developed using B-enriched peripheral blood lymphocytes stimulated with immobilized anti-human IgM and IL-2 [5], and three subgroups are identified as groups A, B and C. Analysis of the groups reveals different patterns of abnormalities in B and T cell phenotypes in the peripheral blood of CVID patients [6], and it also appears that certain clinical features are correlated with the subgroups, for example, splenomegaly with group A [7].

There have been extensive searches for the primary causes of the disease, but common defects have not been identified. It is likely that the immunological defects in CVID involve cells of more than one lineage. B cells from most CVID patients can secrete some immunoglobulins *in vivo* and *in vitro*, whereas B cells that are unable to secrete immunoglobulin under certain experimental conditions can still produce detectable immunoglobulin when transplanted into SCID mice [8–10], supporting a hypothesis that defects in the interactions of T cells and B cells, where adhesion molecules play major roles, may contribute to the primary lesion. Studies by Farrant *et al.* [11] have demonstrated that in four CVID patients examined, B cells in the peripheral blood may be functionally distinct in IgM production following surface IgM cross-linking compared with their splenic counterparts, suggesting dysregulation of B cell trafficking. This may coincide with the poor expression of Leu-8 (L-selectin) on B lymphocytes of some CVID patients [12]. L-selectin is one of the key members of the selectin family of adhesion molecules and plays a critical role in the initiation of leucocyte attachment to activated endothelium [13] and lymphocyte homing [14]. It is constitutively expressed on lymphocytes and neutrophils [15,16]. It has been clearly demonstrated in a transgenic mouse model that

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disruption of L-selectin expression results in severe defects in lymphocyte homing and inflammatory responses [17,18]. L-selectin is shed from the cell surface by proteolytic cleavage of the extracellular region of this molecule following leucocyte activation [19–21]. The shed form can be detected in serum, plasma and other biological fluids [21], with increased levels in diseases such as diabetes mellitus [22], leukaemia [23], adult respiratory distress syndrome [24] and HIV infection [25]. High concentration of sL-selectin can block L-selectin-specific attachment of lymphocytes to the endothelium, and at physiological concentration sL-selectin can also cause a small but consistent inhibition of lymphocyte attachment [21]; thus the levels of sL-selectin in serum may modulate lymphocyte migration across the endothelium. To date, no information is available regarding the levels of sL-selectin in serum or plasma of CVID patients, and L-selectin expression on T cells has not been extensively studied in this disease. The report therefore focuses on a comparative study with normal individuals of L-selectin on the cell surface and in the plasma to assess the contribution of this molecule to possible abnormalities of lymphocyte trafficking in CVID.

MATERIALS AND METHODS

Sample preparation

For flow cytometric analysis, 4 ml of peripheral blood were taken into standard EDTA tubes (containing tripotassium EDTA) from CVID patients attending the immunoglobulin replacement therapy clinic, and from normal individuals (laboratory volunteers and healthy blood donors) as controls. Ethical approval was obtained for these procedures. Plasma samples were derived from these blood samples by centrifugation at 600 g for 15 min at room temperature, and they were stored at -20°C before the assay. For mononuclear cell culture, 20–40 ml of peripheral blood were collected into sterile 20-ml universal tubes containing 200 U of preservative-free heparin. The blood samples were kept at room temperature and processed within 2 h of collection.

Monoclonal antibodies

The following murine MoAbs were obtained commercially in the form of direct fluorescent-conjugates FITC, PE or PerCP where appropriate. Anti-human CD4 (Leu-3a), CD19 (Leu-12), Leu-8 (L-selectin) were purchased from Becton Dickinson; CD21 (BL13), CD45RA (ALB11) from Immunotech; and CD45RO (UCHL-1) from Sigma. Simultest $\gamma 1/\gamma 2$a (Becton Dickinson) was used as an antibody control.

Flow cytometric analysis

Direct, three-colour immunofluorescent staining was used for flow cytometric analysis. In brief, 50 μl of whole blood were treated with 5–10 μl of appropriate antibodies and reacted for 15 min at room temperature in dark. The samples were then treated with 2 ml $1 \times$ FACS Lysing Solution (Becton Dickinson) to lyse the erythrocytes. After two washes with cold PBS + 0·02% azide (4°C) the cells were fixed with 1% formaldehyde in PBS, stored at 4°C and examined on a FACScan flow cytometer (Becton Dickinson) within 24 h. A gate was set on lymphocytes or neutrophils/monocytes and 5000 events were acquired for each sample. The data were analysed using PC-Lysis software (Becton Dickinson) with appropriate gates for each cell population. Results for tested samples were obtained by setting a marker of the positive events in the antibody control sample to <1%. The marker was then copied to the rest of the test samples and the results were expressed as a percentage of the positive events in a defined population.

Patient classification

The principle of the classification method by Bryant *et al.* [5] was adopted. A comparison was initially made using B-enriched peripheral blood mononuclear cells (PBMC) and unfractionated PBMC populations separately in the culture system. It was found that there was a good agreement between patient typing using the two methods (data not shown). Therefore, PBMC culture was used in the subsequent experiments for patient classification. The concentrations of human IgM and IgG in the culture were then measured using a highly sensitive in house ELISA with a detection limit of 0·04 μg/ml for both IgM and IgG. The patients were classified as (i) group A: no IgM or IgG detected; (ii) group B: only IgM detected; and (iii) group C: both IgM and IgG detected in the supernatant. Since group B patients are not numerous, only group A and group C will be referred to separately in the text, where appropriate.

Detection of soluble L-selectin in the plasma

A soluble L-selectin ELISA kit was purchased from Bender MedSystem (Vienna, Austria) for the detection of soluble L-selectin in the plasma. The ELISA kit recognizes both natural and recombinant human sL-selectin in cell culture supernatants, human serum, EDTA or heparinized plasma, etc., with a detection limit of 0·3 ng/ml. Furthermore, there was no cross-reactivity determined for IL-8, soluble intercellular adhesion molecule-1 (sICAM-1), soluble tumour necrosis factor receptor (sTNF-R), TNF-α, CD8, IL-2, IL-2R, IL-6, IL-6R, IL-10, and ELAM-1 (E-selectin), CD44 and HER-2 (manufacturer's data). The experiment was performed following the manufacturer's instructions. The final reaction was read using a Dynatech ELISA reader. The standard absorbency curve was plotted and sample readings calculated using the Reader Manager Software (Dynatech).

Statistical analysis

Statistical analysis of data was performed by analysis of variance in order to determine whether or not there were significant differences at $P < 0{\cdot}05$ level among compared groups. Where such differences existed the minimum significant differences (MSD) were computed at probabilities of 0·05 (MSD0·05) and 0·01 (MSD0·01) using the Tukey–Kramer method [26]. The differences between any pair of means were considered significant if they exceeded MSD0·05, or very significant if they exceeded MSD0·01. Data presented in the text are in the format of mean $\pm$ 1 s.e.m. A n value is also given to represent the number of normal individuals or CVID patients examined in the experiment.

RESULTS

Expression of L-selectin on B cells

As examined using flow cytometry, the great majority of B cells from healthy individuals expressed L-selectin ($90{\cdot}24 \pm 1{\cdot}36\%$, $n = 17$). CVID patients had a significantly lower percentage of L-selectin-positive B cells ($75{\cdot}19 \pm 4{\cdot}64\%$, $n = 21$) compared with normal controls ($P < 0{\cdot}05$). When patient's classification is considered, the reduction in L-selectin-positive B cell population in group A patients ($63{\cdot}00 \pm 4{\cdot}65\%$, $n = 5$) is very significant ($P < 0{\cdot}01$). This is due to the presence of a distinguishable population in group A B cells which is L-selectin-negative,

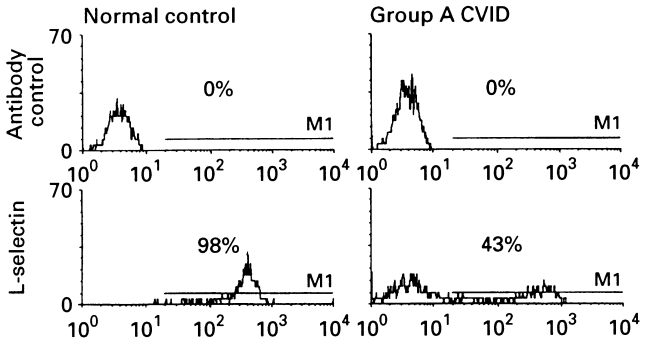

Fig. 1. Flow cytometric histograms of L-selectin expression on B lymphocytes from one normal donor and one CVID patient (group A). Positively stained B cells are marked and indicated with percentages.

whilst the L-selectin-positive B cells express this molecule normally (Fig. 1). No significant difference was found in group C patients compared with normal controls (Fig. 2).

Expression of L-selectin on CD4$^+$ T cells

We also investigated the expression of L-selectin on CD4$^+$ T cells. The two subpopulations of CD45RA$^+$ and CD45RO$^+$ CD4$^+$ T cells were analysed separately. Over 80% of naive CD4$^+$ T cells (CD45RA$^+$) were found to be L-selectin-positive in healthy controls ($n = 18$) as well as in CVID patients ($n = 23$) (Fig. 3). However, a very significant reduction of L-selectin-positive memory CD4$^+$ T cells (CD45RO$^+$) was found in CVID patients ($56{\cdot}77 \pm 3{\cdot}96\%$ for L-selectin-positive, $n = 22$, $P < 0{\cdot}01$), and this reduction was most prominent in group A CVID patients ($34{\cdot}20 \pm 5{\cdot}98\%$ for L-selectin-positive, $n = 5$, $P < 0{\cdot}01$), while no significant difference was found for group C patients ($n = 7$) compared with healthy controls ($76{\cdot}71 \pm 1{\cdot}88\%$ for L-selectin-positive, $n = 17$). CD8$^+$ T cells were not examined in this study.

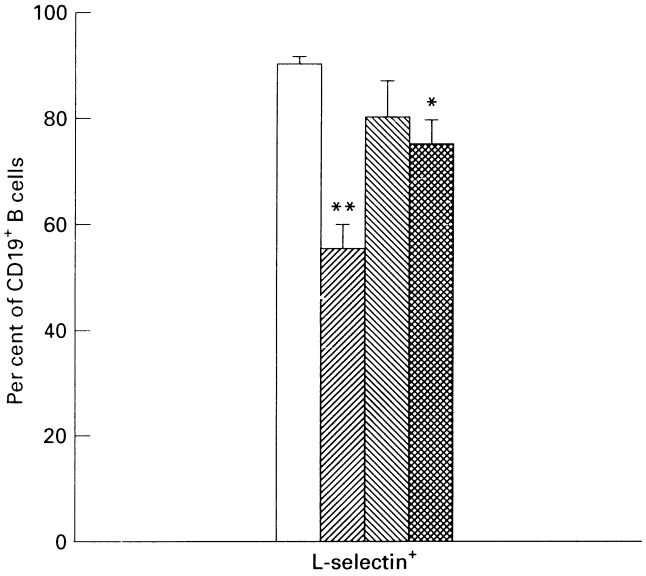

Fig. 2. Expression of L-selectin on B cells in normal controls and the comparative groups of CVID patients. Data are shown as mean + 1 s.e.m. and the comparisons are made with normal controls. □, Normal; ▨, group A; ▧, group C; ▩, pooled CVID. *$P < 0{\cdot}05$; **$P < 0{\cdot}01$.

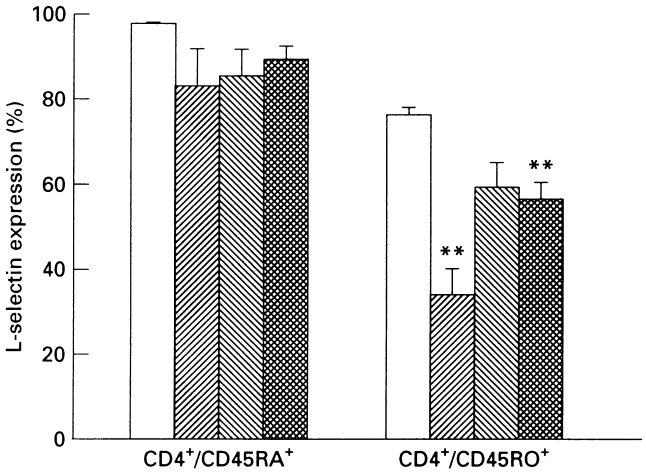

Fig. 3. Expression of L-selectin on the CD45RA$^+$ and CD45RO$^+$ helper T cells. Data are shown as mean + 1 s.e.m. and the comparisons are made with control values. □, Normal; ▨, group A; ▧, group C; ▩, pooled CVID. **$P < 0{\cdot}01$.

Expression of L-selectin on other leucocytes

In order to study the expression of L-selectin on peripheral blood leucocytes other than lymphocytes, an analysis gate was set up to exclude the lymphocyte population which effectively focuses on monocyte and granulocyte populations. A total of eight CVID patients were examined, which included four group A CVID, who have the most severe reduction of L-selectin on their circulating B cells. Over 95% of this non-lymphocyte population from CVID patients expressed high levels of L-selectin on their surface ($97{\cdot}31 \pm 0{\cdot}38\%$, $n = 8$), and no significant difference was found compared with healthy individuals ($98{\cdot}18 \pm 0{\cdot}19\%$, $n = 8$).

Soluble L-selectin in the plasma

Soluble L-selectin was tested in plasma samples from normal individuals and CVID patients using the commercial ELISA kit. An internal positive control was established using sL-selectin standard at 2·5 ng/ml in one dilution to test whether serial dilutions of the standard on the test plate as instructed by the manufacturer would give an accurate result. The tested value for this control is 2·596 ng/ml, an error of <4%.

A panel of 13 plasmas from healthy individuals (male and female) was tested and the detected sL-selectin levels ranged between 374·8 ng/ml and 896·6 ng/ml, with a mean level of 618·9 ng/ml ($618{\cdot}9 \pm 47{\cdot}98$ ng/ml, $n = 13$). A significantly greater value was found in CVID plasmas ($1012{\cdot}4 \pm 57{\cdot}1$ ng/ml, $n = 25$, $P < 0{\cdot}01$), which is 1·6 times the normal value. When subgroups of CVID from the classification were considered, a significantly higher value was found in group A CVID ($983{\cdot}4 \pm 97{\cdot}37$ ng/ml, $n = 8$, $P < 0{\cdot}05$), but not in group C CVID patients' samples ($928{\cdot}1 \pm 87{\cdot}35$ ng/ml, $n = 7$) compared with normal individuals (Fig. 4).

DISCUSSION

This report describes a comparative study of L-selectin on leucocytes and in the plasma of patients with CVID and healthy individuals. The study is in agreement with the earlier observation that B cells from CVID patients have a depressed Leu-8 (L-selectin) expression [12]. Furthermore, when the classification by

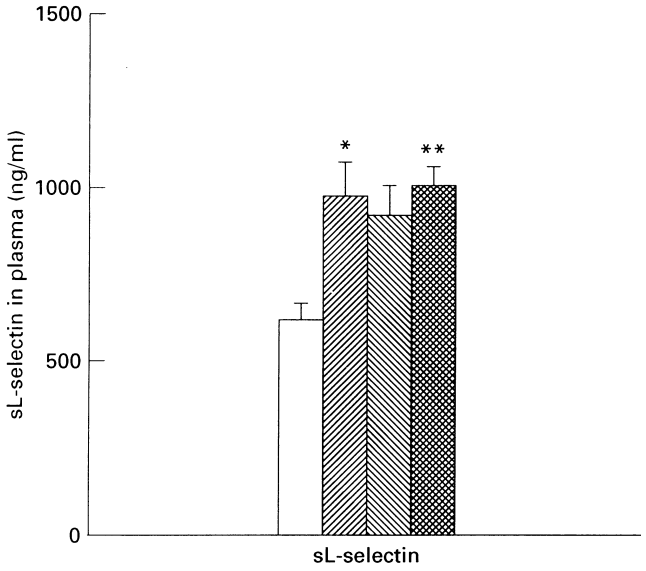

Fig. 4. Soluble L-selectin in plasma. Data are shown as mean + 1 s.e.m. and the comparisons are made with control values. □, Normal; ▨, group A; ▧, group C; ▩, pooled CVID. *$P < 0{\cdot}05$; **$P < 0{\cdot}01$.

Bryant *et al.* is considered, the reduction in L-selectin expression on CVID B cells is marked by a distinctive population of L-selectin-negative B cells in the peripheral blood of group A CVID, and this group of CVID also has very significant reduction in L-selectin-positive B cells compared with normal controls. However, L-selectin-positive B cells in group C were found to be within the normal range. Within the population of $CD4^+$ T cells, the down-regulation of L-selectin expression affected mostly the $CD45RO^+$ population, where the difference between group A and normal controls was highly significant ($P < 0{\cdot}01$). No difference was detected for group C patients compared with controls.

The elevated sL-selectin in serum and plasma has been detected in diseases of many kinds [22–25]. When the normal values are given, they vary from 1·6 to 1·9 μg/ml in serum and 1·25 to 2·1 μg/ml in plasma, as measured using individual reagents. Moreover, sL-selectin is found to be at similar levels in normal serum and plasma [21,25]. Using a commercial ELISA kit, an average value of 618·9 ng/ml ($618{\cdot}9 \pm 47{\cdot}97$ ng/ml, $n = 13$) in the plasma has been found in our 13 healthy individuals in this study. An independent investigation, using the same ELISA kit on circulating sL-selectin in *Plasmodium falciparum* malaria, has established a normal serum level of 658 ng/ml (658 ± 74 ng/ml (mean ± s.e.m.)) from 28 healthy controls [27], a value closely resembling our result. Thus it is likely that the different normal values achieved from our study and that of others [23–25] were due to the different ELISA assays used. Whatever is the case, a significantly elevated sL-selectin level is detected in CVID plasma, approximately at 1·6 times the normal value. This may indicate increased shedding of L-selectin from CVID leucocytes.

L-selectin is constitutively expressed on lymphocytes and neutrophils [15,16] and is lost during cell activation [28] due to proteolytic cleavage at the cell surface [19]. In CVID, over 95% of peripheral blood leucocytes other than lymphocytes express L-selectin at levels similar to that of normal individuals. On $CD4^+$ T cells, the loss of L-selectin is associated with CD45RO expression, a molecule corresponding with T cell memory and activation [29–31]. The loss of L-selectin on this $CD4^+$ T cell subset is likely to be one of the major contributing factors to the elevated plasma sL-selectin levels in CVID. Moreover, the presence of a substantial population of L-selectin-negative B cells in CVID also suggests extensive shedding of this molecule from B cells. Intriguingly, excessive activation of B cells from CVID has not been reported using other markers, and our preliminary observations also indicate that the loss of L-selectin on CVID B cells does not correlate with the activation-related changes of surface IgM and IgD, CD69, CD45RA and CD45RO molecules, indicating a seemingly dysregulated loss of L-selectin on CVID B cells. The absence of surface L-selectin can greatly alter lymphocyte homing and inflammatory responses, and its function can not be replaced by other homing receptors [17,18,32]. Furthermore, it is found that on average 18% of the TCR/CD3 complex associates with Leu-8 (L-selectin), suggesting that L-selectin may also influence or participate in the activation of T cells via the TCR/CD3 complex [33]. On B cells cross-linking of surface L-selectin is found to inhibit immunoglobulin synthesis [34]. Thus, L-selectin may also be involved in cell signalling that affects B cell differentiation and T cell activation [33,34]. In CVID, the unique population of L-selectin-negative B cells in group A patients is virtually absent from the peripheral blood of healthy individuals and group C patients, indicating the existence of a functionally distinct B cell population in this subgroup of CVID patients. Work is currently in progress to delineate the functional capacity of this population and to assess its contribution to the pathology of CVID.

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