Skin-Selective Lymphocyte Homing Mechanisms in the Pathogenesis of Leukemic Cutaneous T-Cell Lymphoma

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The concept of skin-associated lymphoid tissue embraces those cells and functions that are integrated in the cutaneous host defense. Recently, it has been possible to identify those circulating T-cells that are skin associated. These cells display the cell-surface phenotype of memory T cells (CD45RO+) and express the cutaneous lymphocyte antigen, a tissue-selective homing receptor involved in directing T-cell traffic to inflamed skin. To investigate the participation of this skin-associated T-cell subset in the pathogenesis of cutaneous T-cell lymphoma, we studied 16 patients with erythrodermic cutaneous T-cell lymphoma for the presence of these surface proteins on circulating cells. Results were compared with eight patients in remission and eight with minimal patch/plaque cutaneous T-cell lymphoma. The mean expression of both CD45RO and cutaneous lymphocyte antigen were significantly greater in the erythrodermic patients than in the other two patient groups. Expression of these markers was shown to be on the cells of the malignant clone by two-color flow cytometry. These results demonstrate that the malignant cells of cutaneous T-cell lymphoma express the markers of skin homing lymphocytes and that their levels are increased in the erythrodermic cutaneous T-cell lymphoma patients. Moreover, the findings suggest a critical role for the skin-selective homing receptor cutaneous lymphocyte antigen in the pathogenesis of cutaneous T-cell lymphoma. Key words: cutaneous T-cell lymphoma, cutaneous lymphocyte antigen, CD45 isoforms. J Invest Dermatol 101:222–226, 1993

The distribution of the immune repertoire and the functional coordination of spatially distinct compartments of the immune system is critically dependent on a closely regulated system of lymphocyte homing. This system directs naive T lymphocytes to the organized lymphoid microenvironment of secondary lymphoid tissues, such as lymph nodes or Peyer’s patches, in which they can be specifically activated by their specific foreign antigen in conjunction with appropriate accessory cells. Naive T cells (CD45RA+RO-) [1–5] extravasate poorly, if at all, in extralymphoid ("tertiary") sites. In contrast, memory/effecter T cells (CD45RO+) generated in secondary lymphoid tissues in response to stimulation by antigen migrate efficiently to such sites, where they “patrol” for the return of their particular antigen. Memory/effecter T cells do not, however, home equivalently to all possible tissues; instead they demonstrate tissue-selective homing to particular secondary and tertiary sites (reviewed in [1]).

One such site receiving a distinct subset of memory T cells in response to inflammatory stimuli is the skin. Recent studies indicate that T-cell extravasation at cutaneous sites of chronic inflammation is dependent on the interaction of the cutaneous lymphocyte-assoc-

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Abbreviations: CLA, cutaneous lymphocyte antigen; ELAM-1, endothelial-leukocyte adhesion molecule-1; PBML, peripheral blood mononuclear lymphocytes; PE-A, phycoerythrin-conjugated avidin; SALT, skin-associated lymphoid tissue; VB, variable region of the beta chain of the T-cell receptor.
CLA has been demonstrated to be present on the surface of skin-infiltrating lymphocytes in all patients studied with patch-plaque CTCL, but not non-skin-associated T-cell lymphomas [6], suggesting the possibility that the CLA:E-selectin interaction plays a role in the epidermotropic nature of this unique neoplasm. To investigate this hypothesis, we sought to quantify T cells of the skin homing phenotype in the peripheral blood of patients with erythrodermic CTCL, patients with minimal patch/plaque CTCL, and patients with CTCL in remission. Our results indicated that patients with erythrodermic CTCL have increased circulating lymphocytes bearing both CLA and CD45RO. Two of the patients in this study had malignant clones detectable by one of the panel of variable region antibodies. In those patients it can be clearly demonstrated that the circulating CLA^+ and CD45RO^+ cells were malignant cells on the basis of co-expression with the family-specific antibody.

**Table I. Monoclonal Antibodies for Variable Regions of the Human Chain**

<table>
<thead>
<tr>
<th>Name</th>
<th>Immogen</th>
<th>Specificity</th>
</tr>
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<tbody>
<tr>
<td>VB5(a)</td>
<td>Normal donor PBL</td>
<td>VB5.2 + VB5.3</td>
</tr>
<tr>
<td>VB5(b)</td>
<td>Normal donor PBL</td>
<td>VB5.3</td>
</tr>
<tr>
<td>VB5(c)</td>
<td>SUP-T13 T-cell line</td>
<td>VB5.1</td>
</tr>
<tr>
<td>VB6(a)</td>
<td>T-CLL PBL</td>
<td>VB6.7</td>
</tr>
<tr>
<td>VB6(b)</td>
<td>Jurkat T-cell line</td>
<td>VB8</td>
</tr>
<tr>
<td>VB12(a)</td>
<td>Sezary PBL</td>
<td>VB12.1</td>
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**Materials and Methods**

**Patients** A total of 16 patients with erythrodermic CTCL were evaluated in conjunction with their visits to the Dermatology Clinic at Yale University. All had a skin biopsy diagnostic of CTCL (morphologically atypical cells in dermal infiltration and in the epidermis, with Pautrier abscess formation) in conjunction with erythroderma. Eight patients with patch-plaque disease (<10% body surface area) of CTCL were studied, as were eight patients in remission from erythrodermic CTCL at the time of study.

**Preparation of Peripheral Blood Mononuclear Cells** Patients’ blood samples were collected in tubes containing heparin. Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation as follows. Whole blood was diluted with Hank’s balanced salt solution (HBSS) (GIBCO) at a ratio of 3:1. The diluted blood was layered over Ficoll Histopaque-1077 (Sigma Diagnostics) and centrifuged at 300 X g for 35 min at room temperature. The PBMC were harvested from the interface, resuspended, and washed twice in HBSS with 5% fetal calf serum (GIBCO).

**Monoclonal Antibodies** Patients’ PBMC were incubated with a variety of monoclonal antibodies. The anti-CD45RO (UCHL1) was directly conjugated with fluorescein isothiocyanate (FITC) (Dakopatts, Denmark). The unconjugated murine monoclonal antibodies listed in Table I were purchased from T-cell Sciences, Cambridge. The antibody to CLA was directly conjugated with FITC and this was prepared as previously described [6,7]. Mouse ascites fluid was prepared and utilized as previously described [15] to control for non-specific binding of murine antibody in both one-color and two-color studies. The secondary antibody, FITC-conjugated goat anti-mouse IgG, was purchased from The Binding Site, San Diego.

**Immunofluorescence** Isolated PBMC were incubated with primary monoclonal antibody at a concentration of 100,000/100 μl cells for 30 min at 4°C. Phosphate-buffered saline (PBS) mononuclear antibodies were used at concentrations of 0.2 μg/10 μl. After the incubation period, the cells were resuspended and washed two times by centrifugation at 200 X g (4°C) for 5 min with 500 μl PBS (pH 7.4) containing 10% human serum, type AB (GIBCO) and 0.1% sodium azide. FITC-conjugated goat anti-mouse was added to the cells at a concentration of 5 μg/10 μl with 10 μl/100,000 cells used in incubation for 30 min at 4°C. After the secondary staining, the PBMC were washed as stated above.

For two-color immunofluorescence studies, isolated PMLB were initially stained with a monoclonal antibody to the T-cell receptor variable region (either VB5[a] or VB8[a]), which was diluted as described above. The cells were incubated, washed, and resuspended as described in the previous section. The secondary antibody, biotinylated goat anti-mouse IgG, was purchased from Vector Laboratories and the stock solution was diluted 1:200 with PBS. After incubation with the biotinylated antibody, the cells were resuspended and washed two times by centrifugation at 200 X g (4°C) for 5 min with 500 μl/PBS (pH 7.4) containing 10% human serum, type AB (GIBCO) and 0.1% sodium azide. Following the last washing step, the cells were stained with phycoerythrin-conjugated avidin (PE-A) (CALTAG Laboratories), which was diluted with PBS to a concentration of 1:10. After a 30-min incubation, cells were washed twice as described above and then incubated with the FITC-conjugated monoclonal antibody to CD45RO or CLA.

**Flow Cytometry Analysis** For flow cytometry analysis, the cells were resuspended in a final volume of 500 μl PBS. A cytofluorograph IFA (Ortho Diagnostics Systems Inc, Westwood, MA) with an Argon laser (Exel, Westwood, MA) was used for fluorescence analysis. Gating was performed on lymphocytes and background fluorescence was set at 5%. The percentage of positive cells were obtained by subtracting 5% from the percent expressing cells. For two-color flow cytometry, red and green background fluorescence was set at 5%. The two-color flow cytograms were divided into four quadrants based on each line being drawn at 5% control immunofluorescence. The percentage of cells in each quadrant is displayed on the cytogram. Results were thus expressed as a percentage of total lymphocytes. Two-color flow cytometry was performed on a Dual Laser FACS IV (Becton Dickinson).

**Statistical Analysis** The correlation coefficient (r) was used to measure the strength of the linear association between the flow cytometric results with CD45RO and CLA. Student’s t test was used to test for differences between the study groups: p < 0.05 was considered significant.

**Results**

**CD45RO Expression** The results of memory cell analysis on PBMCs from CTCL patients in remission and those with limited cutaneous disease were similar to those reported for normal controls [4]. In the 16 non-leukemic patients, CD45RO was expressed on a mean of 15% of total lymphocytes in the eight in remission, and a mean of 13.5% of total lymphocytes in the eight patients with patch-plaque disease. None of the patients in remission or with limited patch-plaque disease had greater than 33% of peripheral blood lymphocytes expressing CD45RO, whereas most of the leukemic patients were above this level, as shown in Fig 1.

In the leukemic CTCL group CD45RO expression was markedly elevated, with a mean of 56% of total lymphocytes, which was significantly higher than that seen in the non-leukemic patients (p < 0.05).

**Coexpression of CD45RO and TCR VB Epitopes** Of all 16 leukemic patients screened with the panel of the variable region of the beta chain of the T-cell receptor (VB) antibodies in Table I, only two patients were identified with elevated levels of circulating cells expressing a particular VB. Normally, less than 5% of circulating cells express a given VB. One patient had circulating cells expressing 77% VB8; 4 weeks later this patient had 84% VB8 expressing cells. The second patient had 93% VB5 expressing cells. Two-color flow cytometry demonstrated that CD45RO is expressed on the malignant clone. In Fig 2, the percentage of CD45RO^+VB8^+ cells in that patient was 67% whereas only 4% were CD45RO^+VB8^ and 11% were CD45RO^+VB8^.

**CLA Expression** The results of CLA expression analysis on PBMCs from CTCL patients in remission and those with limited cutaneous disease were similar to those reported for normal controls [6]. The percentage of lymphocytes bearing CLA in the 16 non-leukemic patients showed a mean of 15.5 for the eight in remission, and a mean of 14.5 for the eight patients with patch-plaque disease. None of the limited patch-plaque disease patients or those in remission had greater than 21% of peripheral blood lymphocytes expressing CLA, as shown in Fig 3. In two of the leukemic CTCL group, CLA expression was significantly elevated, being expressed on a mean of 52% of peripheral blood lymphocytes (p < 0.01 compared to non-leukemic patients). In addition, 14 of the 16 patients had >21% of peripheral blood lymphocytes expressing CLA.

**Coexpression of CLA and TCR VB Epitopes** In the results presented above it was reported that VB antibody screening of peripheral blood lymphocytes found that one patient’s peripheral blood lymphocytes expressed the epitope recognized by VB5(a) and
one recognized by VB8(a). Two-color flow cytometry with the VB antibodies demonstrated that CLA is expressed on the malignant clone (Fig 4A,B). The patient sample shown in Fig 4A was the same patient as in Fig 2 at a later date, when 84% of the peripheral blood lymphocytes were expressing VB8. In Fig 4A, 52% of the circulating lymphocytes were CLA+VB8+; however, 32% of the lymphocytes expressed VB8 but did not express CLA. The patient sample in Fig 4B was characterized by having 93% of the lymphocytes expressing the VB epitope recognized by antibody VB5(a). As shown in Fig 4B, a large number of clonally expanded VB5(a)+ cells are CLA+ (52%). As with the patient sample in Fig 4A, there were VB+ and CLA+ peripheral blood lymphocytes (41%).

Correlation of CD45RO and CLA In Fig 5, each patient analyzed is represented by a single point whose position was plotted as a dual function of the percentage of CD45RO+ lymphocytes (y-axis) and the percentage of CLA+ lymphocytes (x-axis). As can be seen in Fig 5, each patient is represented by a single point. There was a positive correlation between CD45RO and CLA expression with a correlation coefficient r = 0.70 (p < 0.05).

**DISCUSSION**

The concept that the skin constitutes a specific immunologic microenvironment is not a new one [16,17], but has received a great deal of support in recent years from studies indicating that unique constituents of the skin such as Langerhan’s cells, keratinocytes, and melanocytes can extensively interact with lymphocytes, either by secretion of immunoreactive cytokines or by expression of cell adhesion/recognition receptors (e.g., ICAM-1, HLA-DR) that mediate direct cell–cell contact [18]. Moreover, it is now clear that the lymphocytes entering the skin are themselves a unique subpopulation, and that the skin represents a distinct lymphocyte homing specificity [6,7]. Skin-associated lymphocytes are primarily T cells that display the CD45RO+ phenotype of memory T cells [19], and selectively express the cell-surface determinant CLA [6]. CLA is an oligosaccharide ligand for E-selectin, a vascular adhesion molecule that in the setting of chronic inflammation is preferentially expressed in skin, and it is thought that the CLA:E-selectin interaction is a major determinant of the skin-selective T-cell homing specificity [1,6-9].

The clinical syndromes made up of certain T cell malignancies localizing to the skin have been collectively known as CTCL [10,20,21]. The existence of these skin homing lymphomas has long been used as an argument for a skin-selective homing specificity in man. Now, with the recognition that there is a physiologic, skin-associated T-cell subset, it has become possible to address this issue from the reverse perspective, that of defining the cellular and molecular mechanisms underlying the distinct pathophysiology of these neoplasms. In this report, we demonstrate that patients with erythrodermic CTCL have in their peripheral blood elevated levels of cells expressing the memory marker CD45RO and the skin-homing receptor CLA. Moreover, in patients whose malignant clone could be unequivocally identified with monoclonal antibodies against T-cell receptor variable region epitopes, the predominant phenotype of the malignant population was CD45RO+, CLA+. These results are in keeping with a previous study demonstrating the CTCL leukemic cells preferentially home to skin [22], and are consistent with studies showing that the malignant skin infiltrates of patch/plaque-stage mycosis fungoides have a memory T-cell phenotype [23], and are predominantly CLA+ [6]. As CLA expression is not a feature of T-cell lymphomas in extracutaneous sites [6], these observations suggest that the variants of CTCL that have appeared under the colorful names of mycosis fungoides, Pagetoid reticulosis, Sezary syndrome, etc. can now be unified by the concept that they are malignancies of cutaneous T cells (skin-homing memory T cells). The studies of CLA expression also suggest that the same mechanisms directing the normal skin-selective homing of this subset may underlie the distinctive skin localization of these neoplasms.

Recent advances in the understanding of the normal regulation of
Figure 3. CLA expression on total peripheral blood lymphocytes in CTCL patients. On the left (A) are eight patients in remission from CTCL (mean 15.5%). In the center (B) are eight patients with patch-plaque disease (<10% body surface area) with a mean of 14.5%. The 16 leukemic patients (C) on the right had a mean of 52% CLA+ cells (p < 0.05 versus the other two groups).

Figure 4. (A) Two-color flow cytograms from two CTCL patients showing CLA expression on the x-axis and TCR VB expression on the y-axis (VB5(a) for the patient in A, VB5(a) for the patient in B). The lines dividing the plots into quadrants intersect an axis at the fluorescence channel where <5% of cells had greater fluorescence with control staining. The percentages of cells in each quadrant are listed in the quadrant.

CD45 isoforms and CLA in physiologic situations suggest provocative hypotheses on the origins and pathogenesis of CTCL. Virgin T cells emigrating from the thymus or recirculating among secondary lymphoid tissues have a CD45RA+/CLA- phenotype. Activation of these virgin T cells in secondary lymphoid tissues leads to the replacement of CD45RA by the RO isoform, and, depending on the local microenvironment, upregulation of CLA (CLA upregulation is over five times more frequent in peripheral lymph nodes draining skin than in non-skin-associated secondary lymphoid tissues such as the appendix [24,25]). Cutaneous T cells obtained from suction blisters overlying delayed hypersensitivity reactions are almost entirely CD45RO+ [19], and CLA+ (the mean CLA expression of cutaneous T cells is 23 times higher than that of peripheral blood T cells [25]). Because malignant transformation likely occurs or becomes manifest in a proliferating cell, the finding that CTCL cells are CD45RO+/CLA+ suggests that malignant T-cell clones of CTCL arise either in T cells undergoing the virgin-to-memory transition in secondary lymphoid tissues (most likely peripheral lymph node) or in CLA+ memory T cells activated in the skin. Thus, it is likely that at the time of transformation, CTCL clones have "seen" antigen and undergone antigen-driven activation [26].

The microenvironment of this activation is presumably favorable to the upregulation of the CLA epitope. Furthermore, it is possible that the maintenance of CLA expression on the malignant clone requires continued re-stimulation in an appropriate microenvironment. The finding that CTCL cells often express a T-cell receptor-mediated activation marker (BE2) is in keeping with this hypothesis [15,27]. A requirement for continuous CLA upregulation might explain the finding that not all circulating CTCL cells are CLA+ (Fig 4). The CLA+ and CLA- subsets might reflect cells recently exposed to the cutaneous or other appropriate microenvironment versus those not receiving such CLA-inducing signals. Another explanation for the VB+CLA- cells would be that these are tumor cells in the process of dedifferentiation. Followup studies will determine if this is a sign of tumor progression.

Clinical progression of CTCL is associated with loss of epidermotropism and widespread dissemination [10]. It is possible that loss of CLA expression with tumor dedifferentiation plays a role in this process. Such loss may occur directly, with loss of expression of the core polypeptide of CLA or the glycosyl transferases required to produce its unique oligosaccharide side chain, or may occur indirectly by virtue of the tumor losing the ability to respond to CLA-inducing stimuli. Preliminary observations suggesting that development of (non-epidermotropic) tumor stage CTCL is associated with (and perhaps preceded by) loss of CLA expression [6] support this hypothesis.

Previous studies have suggested the potential utility of T-cell receptor variable region-specific antibodies in the diagnosis and monitoring of T-cell malignancies [14]; the results of this study confirm this potential. Unfortunately, the current paucity of rea-
The correlation of CD45RO expression with CLA expression in CTCL patients. All patients in this study were plotted as a point determined to demonstrate the positive correlation (r = 0.70, p < 0.001).

REFERENCES


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