Skewed Expression of Activation, Differentiation and Homing-Related Antigens in Circulating Cells from Patients with Cutaneous T Cell Lymphoma Associated with $CD7^-$ T Helper Lymphocytes Expansion

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Mycosis fungoides and Sézary syndrome represent the most frequent forms of cutaneous T cell lymphoma. Both are characterized by skin infiltrating and/or circulating malignant cells displaying a $CD4^+CD7^-$ phenotype in the majority of cases. Because an expansion of $CD4^+CD7^-$ cells may also be found in inflammatory dermatoses or in the aging process, we evaluated, by flow cytometry, the relationship between CD7 expression and the distribution of differentiation/activation or homing antigens on peripheral blood lymphocytes from 36 cutaneous T cell lymphoma patients and from healthy donors. $CD4^+CD7^-$ cells were increased in all patients with cutaneous T cell lymphoma. As a consequence, the $CD7^{+/-}$ ratio was reduced in stage I–II mycosis fungoides (3.96 vs 6.55 in healthy donors), and inverted in stage III–IV MF and Sézary syndrome (0.28 and 0.12 respectively). In the late stage of disease, the $CD7^{+/-}$ inverted ratio was strictly related to the expression of CD15s, CD60, and CD45R0, and the lack of expression of CD26 and CD49d. Interestingly, in leukemic patients, this phenotype was also associated with peculiar morphologic (large size) or phenotypical ($CD3^{dim}$ expression) characteristics. Furthermore, a progressive reduction of circulating $CD8^+$ cells was also seen throughout all stages of disease. The presence of these populations in cutaneous T cell lymphoma at late phases of disease and Sézary syndrome suggests that all of these molecules may play an important part in the activation pathway and skin homing of circulating T cells in lymphoproliferative disorders. Therefore, this may constitute a distinctive feature in cutaneous T cell lymphoma patients with more aggressive characteristics. ***Key words: CD15s/CD26/CD45R0/CD60, VLA4.***
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Cutaneous T cell lymphomas (CTCL) comprise a heterogeneous group of lymphoproliferative disorders primarily affecting the skin. Mycosis fungoides (MF) and its leukemic variant, Sézary syndrome (SzS) are the most frequent manifestations. MF is an indolent cutaneous lymphoma characterized by slow progression and a long survival, usually manifesting with flat patches or infiltrated plaques. After a variable period of time, an evolution to skin nodules and the spread to lymph nodes or other visceral sites may occur. SzS is a more aggressive form, associated with extracutaneous involvement and characterized by erythroderma, lymphadenopathy, and circulating T helper malignant cells with cerebriform nuclei (Diamandiou *et al*, 1996; Willemze *et al*, 1997).

Two main features characterize these cells. First, there is a CD7 negative phenotype in approximately 75% of patients (Haynes *et al*, 1981; Berger *et al*, 1982; Wood *et al*, 1986; Ladastide *et al*, 1990; Harmon *et al*, 1996; Bogen *et al*, 1996). Second, there is a clear predilection for skin homing (Picker *et al*, 1990). Thymocytes early during ontogeny and normal circulating T cells express CD7, a 40 kDa glycoprotein member of the immunoglobulin gene superfamily (Lobach *et al*, 1985). CD7 negative T cells are normally found in the peripheral blood at a low percentage (Reinhold *et al*, 1993), but an increase can be seen in the aging (Kukel *et al*, 1994) and in a number of pathologic conditions, such as severe combined immunodeficiency or inflammatory skin disease (Jung *et al*, 1986; Moll *et al*, 1994). Recent studies suggest the involvement of this antigen both in signal transduction and modulation of T lymphocyte adhesive interactions (Shimizu *et al*, 1992). It is also known that repeated mitogenic stimulation of naive $CD4^+$ cells from normal individuals leads to the development of two memory subsets, defined by the presence/absence of the CD7 antigen (Reinhold *et al*, 1996). CD7 negative cells preferentially express CD60, a 9-*O*-acetylated form of GD3 ganglioside found on 30–60% of peripheral memory T lymphocytes, malignant T cells, some cell lines and IL-4 producing cells (Kniep *et al*, 1993), and the cutaneous lymphocyte-associated antigen (CLA). On the other hand, the $CD7^+$ T cell subset shows an enhanced percentage of the CD27 antigen, a 100 kDa transmembrane glycoprotein belonging to the tumor necrosis factor receptor (TNF-R) family, which provides

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Abbreviations: CLA, cutaneous lymphocyte antigen; CTCL, cutaneous T cell lymphoma; MF, mycosis fungoides; SzS, Sézary syndrome.

costimulatory signals for T cell activation (Hintzen *et al*, 1993). Both $CD7^+$ and $CD7^-$ T cells express similar levels of several activation/differentiation antigens such as CD26, CD28, and CD45R0 (Reinhold *et al*, 1996). CD26, the T cell's activation antigen dipeptidyl peptidase IV, is a 100 kDa glycoprotein that may also interact with the extracellular matrix proteins fibronectin or collagen (Kameoka *et al*, 1993). CD28 and CD7, which belong to the immunoglobulin gene superfamily, are critically involved in T cell co-stimulation, and provide comitogenic activation signals for T cells (Koulova *et al*, 1991). Furthermore, cross-linking of CD7 and CD28 surface antigens modulates T cell adhesion (Shimizu *et al*, 1992). CD45, a transmembrane glycoprotein with phosphatase activity, has been proposed as a marker to discriminate memory (expressing CD45R0 isoform) from unprimed (CD45RA) T cells (Streuli *et al*, 1987; Akbar *et al*, 1988). Taken together, these data indicate an important relationship between CD7 and these activation/differentiation markers. Their combined study might help with the identification of important cell subpopulations for several disease statuses.

Another characteristic of leukemic cells in CTCL is their commitment to skin homing (Heald *et al*, 1993). Trafficking of T lymphocytes to the skin is mediated by the interaction of carbohydrate ligands such as CLA and CD15s on the T cell surface with E- or P-selectin expressed by dermal vascular endothelium (Fuhlbrigge *et al*, 1997; Tietz *et al*, 1998). CLA is a fucosyltransferase VII-mediated modification of P-selectin glycoprotein ligand-1 which allows binding to E-selectin (Picker *et al*, 1990; Fuhlbrigge *et al*, 1997). Also, T cells expressing the variant type of sialyl Le_x (CD15s) defined by 2F3 monoclonal antibody (MoAb), show a clear E-selectin-dependent adhesion to activated endothelial cells and are usually committed to the skin (Ohmori *et al*, 1993; Furukawa *et al*, 1994). The integrin VLA4 (CD49d) modulates the migration of the lymphocyte through the vessel wall after binding to VCAM-1 (cell adhesion molecule expressed on activated endothelium). VLA4 also mediates the firm adhesion to the subendothelial extracellular matrix and therefore is directly involved in the skin homing of T lymphocytes (Savoia *et al*, 1992; Springer, 1994).

Based on these considerations, we investigated the correlation between the expression of CD7 and the distribution of differentiation/activation antigens or homing-related molecules in peripheral blood lymphocytes (PBL) from CTCL patients at various disease stages and in SzS cases.

MATERIALS AND METHODS

Patients Thirty-six patients (25 males, 11 females, mean age 64.9±18.8 y) with CTCL were included in the study. **Table I** summarizes the sex, age, clinical stage, lymphomonocyte, and Sézary cell count in each patient. Eighteen healthy individuals (12 men, six women, mean age 50.6±8.4 y) acted as normal controls. The diagnosis was based on physical examination, histology, and immunohistology. Quantitation of Sézary cells from each patient was performed on blood film using Wright's stain. Staging was based on TNM criteria (Bunn *et al*, 1979). Five adult patients (four males and one female) with severe atopic dermatitis and seven patients (five males and two females) suffering from moderate to severe plaque-type psoriasis (affected body surface areas ranging from 30 to 60%) were included as inflammatory dermatoses controls. Systemic treatments were discontinued in all patients studied at least 15 d before blood specimens were collected.

Three-color flow cytometry analysis In this study the following MoAb were used: unconjugated rat anti-human CLA (PharMingen, San Diego, CA); fluorescein isothiocyanate (FITC)-conjugated anti-CD7, CD11a, CD15s, CD27, CD30, CD45R0, CD54, CD60, and CD95; R-Phycoerythrin (PE) conjugated anti-CD26, CD28, CD45RA, CD49d (VLA4), and CD69; FITC, PE, or peridin chlorophyll protein (PCP) conjugated anti-CD3, CD4, or CD8 (Becton Dickinson Immunocytometry System, Mountain View, CA). Three-color flow cytometry analysis was performed as previously described (Scala *et al*, 1988). Briefly, fresh peripheral venous blood samples from patients with CTCL and controls were collected in tubes containing 1.5 mg of ethylenediamine tetraacetic acid-κ_2 per ml of blood. One hundred microliters of well-mixed whole blood was delivered into the test-tube and placed in an ice-water bath. Erythrocytes were lysed according to the manufacturer's instructions using ammonium chloride lysing solution (Ortho-mune lysing solution; Ortho Diagnostic System, Raritan, NJ) for 10 min at room temperature. PBL were washed and resuspended in cold phosphate-buffered saline (PBS) containing 2% fetal bovine serum (FBS; GIBCO, Paisley, Scotland, U.K.) and 0.02% natrium azide (NaN_3) at 2×10^6 per ml. All the subsequent steps were completed while keeping the cells in an ice bath. A total of 100 μl of cell suspension was mixed with 10 μl of each MoAb and incubated in the dark for 30 min at 4°C. After 30 min, cells were washed twice in cold PBS and then resuspended in 300 μl of PBS containing FBS and NaN_3 for immediate flow cytometry analysis. An irrelevant mouse IgG1 MoAb (Becton Dickinson) was used as a negative control.

For the determination of CLA positive cells, 100 μl of cell suspension was mixed with 4 μl of HECA-452 (rat IgM MoAb to CLA, PharMingen) and incubated at 4°C for 30 min. After two washes, 4 μl of PE-rabbit anti-rat IgG was added and incubated in the dark at 4°C for 30 min. After two additional washes, 4 μl of normal mouse serum was added to each sample and after 10 min the other two MoAbs (PcP-CD3 and FITC-CD4) were added. After 30 min of incubation in the dark at 4°C, cells were washed twice and resuspended in 300 ml of PBS containing FBS and NaN_3 for immediate flow cytometry analysis. Staining with an irrelevant rat IgM MoAb (Pharmingen) was used as a control.

Quantitative analysis for three-color flow cytometry was carried out using an FAC-Scan instrument (Becton Dickinson). At least 10,000 events were acquired in list mode, and all the data were analyzed by the CellQuest software (Becton Dickinson). Live lymphomonocytes were electronically gated by forward and right angle scatters.

Statistical analysis Statistical analysis was performed using SigmaStat version 1.0 software (Jandel Corporation, San Rafael, CA). Differences between percentages and absolute numbers were completed using the Mann–Whitney U test and the Spearman rank correlation coefficient. The tied p-values are provided.

Table I

	Age/sex	Clinical stage	Lymphomonocyte cell count per μl	Sézary cell count per μl
1	65/F	IA	1020	<10
2	65/M	IA	1500	<10
3	67/M	IA	1500	<10
4	75/F	IA	1270	<10
5	75/M	IB	1300	<10
6	52/M	IB	1470	<10
7	59/M	IB	2109	<10
8	70/M	IB	1050	<10
9	60/F	IB	746	<10
10	68/M	IB	2540	<10
11	87/M	IIA	745	<10
12	72/M	IIA	489	<10
13	62/M	IIA	1840	<10
14	63/F	IIA	2269	<10
15	65/M	IIA	1952	<10
16	64/M	IIA	1155	<10
17	78/F	IIB	1927	<10
18	61/M	IIB	1630	<10
19	75/M	IIB	1013	<10
20	48/M	IIB	267	<10
21	66/M	IIB	1224	<10
22	65/M	IIB	493	<10
23	40/M	IIIA	1350	12
24	65/F	IIIA	880	17
25	58/F	IIIA	6129	367
26	38/F	IIIB	1667	33
27	68/M	IVA	1500	77
28	69/F	IVA	2845	113
29	66/M	IVB	12 620	378
30	87/F	IVB	1200	12
31	78/M	SzS	8800	5984
32	68/M	SzS	4935	2072
33	59/M	SzS	3640	3094
34	54/M	SzS	4646	2462
35	69/M	SzS	3321	2285
36	61/F	SzS	9780	7628

Table II

	$CD3^+$		$CD3^+$				$CD3^+CD4^+$			
	%	Absolute no.	$CD4^+$		$CD8^+$		$CD7^+$		$CD7^-$	
			%	Absolute no.	%	Absolute no.	%	Absolute no.	%	Absolute no.
Healthy donors (18)										
Mean±SD	72.5±8.2	1212±169	59.6±8.8	725±142	32.8±8	401±116	86.7±7.2	655±106	13.2±7.3	97±49
MF [I–II] (22)										
Mean±SD	77.5±8.5	1048±526	67.1±14.3	730±464	25.6±10.8	245±149	80.06±11.5	589±404	20.2±11.1	143±77
Mann–Whitney U test	—	—	—	—	$p<0.03$	$p<0.002$	—	—	—	—
MF [III–IV] (8)										
Mean±SD	88.8±5.3	2577±1616	88±14.6	2388±1627	9.11±9.4	156±114	21.9±17.8	522±612	77.3±17.9	2150±1438
Mann–Whitney U test	$p<0.0001$	$p<0.03$	$p<0.0001$	$p<0.05$	$p<0.0001$	$p<0.0001$	$p<0.0001$	—	$p<0.0001$	$p<0.0001$
SzS (6)										
Mean±SD	85.5±14.4	5966±4053	89.6±10.2	5412±3976	8.3±9.6	427±540	11.2±5.2	433±244	88.7±23.6	3691±2589
Mann–Whitney U test	$p<0.02$	$p<0.002$	$p<0.0001$	$p<0.0001$	$p<0.0001$	—	$p<0.0001$	—	$p<0.0001$	$p<0.005$

RESULTS

Differentiation/activation markers We evaluated the expression of several differentiation/activation markers on circulating $CD4^+$ T cells from 36 patients with CTCL (**Table I**) and 18 healthy individuals and compared the results.

The $CD3^+CD4^+$ lymphocyte percentage was slightly increased in 22 patients with the early stages of disease but markedly increased in the advanced stages of MF (eight cases, four patients having diffuse erythroderma and four at the tumor stage) and SzS (six cases). In addition, the absolute $CD3^+CD4^+$ lymphocyte count in the later stages of MF and in SzS was increased compared with controls (**Table II**). Also, the percentage and absolute number of $CD3^+CD8^+$ lymphocytes was lower in stage I and II patients with MF, and even more decreased in the late stage of MF when compared with healthy donors. Therefore, a significant inverse relationship between $CD3^+CD4^+$ cells and $CD3^+CD8^+$ cells was observed (r_s –0.83, $p<0.001$). Surprisingly, the absolute number of $CD8^+$ cells was not different between patients with SzS and controls (**Table II**).

In normal donors, $CD7^+$ lymphocytes were about 80% of total T helper cells ($CD7^{+/-}$ ratio: 6.55). Also, in MF cases in the plaque–patch stage, $CD7^+$ lymphocytes outnumbered $CD7^-$ lymphocytes, but a decrease of the $CD7^{+/-}$ ratio (3.96) was noted. On the contrary, in the late stages of MF and in SzS patients, an inversion of the $CD7^{+/-}$ ratio occurred (0.28 and 0.12, respectively). The inversion of the $CD7^{+/-}$ ratio in these patients was mainly due to a significant expansion of both the percentages and the absolute number of CD7 negative cells ($p=0.0001$ by Mann–Whitney U test). This increase was also observed, but at a much lower level, in the earliest phase of disease (**Table II**). No age differences were present between MF patients at late stage and controls, whereas both MF patients at early phases of disease and SzS cases were slightly older than healthy donors (**Table I**). We observed only a slight, but not significant, increment of circulating CD7 negative cells in psoriasis patients and no difference was observed in atopic dermatitis patients (data not shown).

We then studied the expression of several other molecules such as CD26, CD27, CD28, CD30, CD45R, CD60, CD69, and CD95 on $CD4^+$ T cells. As shown in **Table III**, no significant differences in expression were noted comparing patients with MF at the plaque–patch stage and healthy individuals. On the other hand, we could detect a significant increase both in the percentage and the absolute number of $CD4^+$ cells expressing CD60 and CD45R0 but lacking CD26 and CD45RA surface antigens in patients in the late stages of MF and SzS patients (**Table III**). No significant differences in expression of any of these markers between SzS cases and MF patients with stage III or IV disease were ever seen. Notably, the direct correlation between the percentage of CD7 negative cells and all of the antigens mentioned was observed (**Table IV**). These data suggest that the coexpression of CD60 and CD45R0 antigens in CD7/CD26 double negative cells might be a distinctive feature of patients with CTCL in late stages of disease.

Among the other differentiation/activation markers studied, only a small percentage increase ($p<0.05$) of $CD27^+$ T helper cells in patients compared with controls was observed (64.1±19.2 in healthy donors vs. 74.7±19.7 in the early stages of MF, 80.8±13.9 in the late stages of MF and 81.9±11.6 in SzS cases). No significant difference in expression of CD30, CD69, and CD95 between healthy donors and patients with CTCL was observed; however, an abnormal expansion of CD28 negative cells in two patients with SzS (50% and 39% of total $CD4^+$ cells respectively) and one patient with stage IV MF (61% of total $CD4^+$ cells) was found. All mentioned markers were not statistically different comparing patients with psoriasis and atopic dermatitis to healthy individuals (data not shown).

Homing-related molecules When we looked at the expression of homing-related antigens, such as CD11a, CD15s, CD54, and VLA4 within $CD4^+$ T cells, no difference between normal donors and patients with stage I or II MF was observed (**Table III**). Interestingly, patients with late stages of MF and patients with SzS showed a significant increase in the number of $CD4^+$ T lymphocytes expressing CD15s but lacking VLA4 (CD49d) (**Table III**). A direct relationship between the percentage of CD7 negative cells and the percentage of $VLA4^-$ and $CD15s^+$ lymphocytes was also found (**Table IV**). No significant differences in the expression of CD11a and CD54 were found between our study groups (data not shown). As previously suggested for differentiation/activation molecules, these data led us to hypothesize that an atypical population of CD7/VLA4 double negative but CD15s expressing T lymphocytes might be present in the late stages of CTCL.

As it has been reported that CLA is increased in lymphoproliferative skin disorders (Picker *et al*, 1990), we also evaluated the expression of this antigen. 68.2 ± 18.1% of $CD4^+$ T lymphocytes from patients with late stages of MF express CLA. In addition, a coexpression of CLA and CD15s was also found (**Fig 1**).

T cell subset distribution in patients with SzS We then wondered if further differences in T cell antigen expression could be detected between patients with late stages of MF and SzS. Surprisingly, no significant differences were noted in the expression of the molecules we studied, with the exception of CD49d expression on $CD4^+$ T cells (18.7±6.3% in SzS vs. 38.6±18.1% in the late stages of MF, $p<0.03$ by Mann–Whitney U test).

When we looked at the intensity of CD3 expression, three patients with SzS showed a bimodal distribution of this antigen, allowing for a discrimination between bright (normal) or dim (atypical) intensity (**Fig 2**) as previously described (Bogen *et al*, 1996). We found that the percentage of $CD3^{dim}$ cells constantly outnumbered the "bright" cell population. Interestingly, $CD3^{dim}$ cells expressed CD60 but not VLA4 or CD7. On the other hand,

Table III. Differentiation/activation and homing-related molecules within peripheral blood $CD4^+$ T cells

	$CD60^+$		$CD26^-$		$CD45R0^+$		$CD15s^+$		$CD49d^-$	
	%	Absolute no.	%	Absolute no.	%	Absolute no.	%	Absolute no.	%	Absolute no.
Healthy donors (18)										
Mean±SD	23.6±11.3	301±144	32.13±13.4	381±175	52.4±13.4	408±91	23.44±8.7	176±57	25.3±17.01	371±167
MF [I–II] (22)										
Mean±SD	20.5±9.9	204±189	42.4±21.06	473±460	55.1±19.03	385±341	25.9±10.06	168±108	23.6±19.1	291±442
Mann–Whitney U test	—	—	—	—	—	—	—	—	—	—
MF [III–IV] (8)										
Mean±SD	54.8±15.1	1561±1181	92.1±3.7	2671±1467	78.8±25	2285±2142	65.5±22.6	1723±1354	63.1±19.03	1826±1347
Mann–Whitney U test	$p<0.0001$	$p<0.006$	$p<0.0001$	$p<0.0001$	$p<0.01$	$p<0.0001$	$p<0.003$	$p<0.003$	$p<0.0002$	$p<0.02$
SzS (6)										
Mean±SD	67.6±21.2	4585±4283	93.7±5.1	5305±2715	87.6±12.01	3695±2325	79.9±23.6	3889±2414	81.2±17.25	4868±3838
Mann–Whitney U test	$p<0.0001$	$p<0.0001$	$p<0.0001$	$p<0.0001$	$p<0.0001$	$p<0.0001$	$p<0.0001$	$p<0.006$	$p<0.0001$	$p<0.0001$

Table IV. Spearman rank order correlation between CD7 negative cell and activation, differentiation, or homing-related antigens percentages

	Correlation coefficient (r_s)	p-value
$CD60^+$	0.75	<0.001
$CD45R0^+$	0.42	<0.03
$CD15s^+$	0.67	0.003
$CD26^-$	0.74	<0.001
$CD45RA^+$	−0.45	<0.01
$CD49d^-$	−0.49	0.02

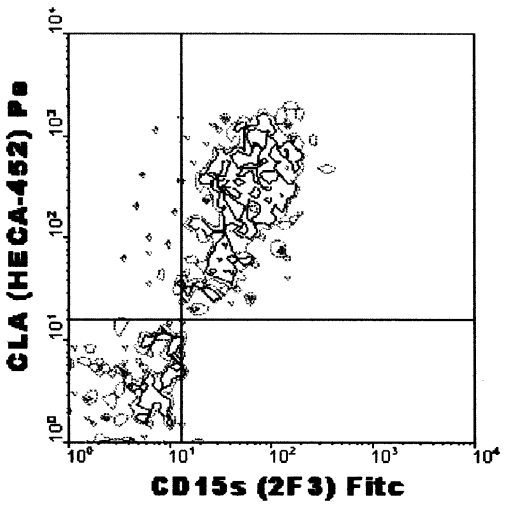

Figure 1. Electronically gated $CD4^+$ cells coexpress CD15s and CLA antigens.

all $CD3^{bright}$ cells were mostly $VLA4^+$ and $CD7^+$ but not $CD60^-$ (**Fig 2**). In the remaining three patients with SzS, two lymphoid populations were detected on the basis of cell size. In these cases the larger sized (lymphoblastic) population was almost exclusively $CD3^+CD4^+CD60^+CD7^-$ and $VLA4^-$, resembling the phenotype of the $CD3^{dim}$ T cells previously described. The smaller sized subset of lymphocytes were mostly $CD7^+$, $CD60^-$ and $VLA4^+$. None of these populations ($CD3^{dim}$ or lymphoblastic T cells) were ever observed in other patients with MF or in healthy controls.

DISCUSSION

In this study we investigated the expression of several activation and homing surface antigens on PBL in a cohort of 36 patients with CTCL. Within the $CD4^+$ population of T cells, the number of circulating lymphocytes expressing CD7 from patients with the

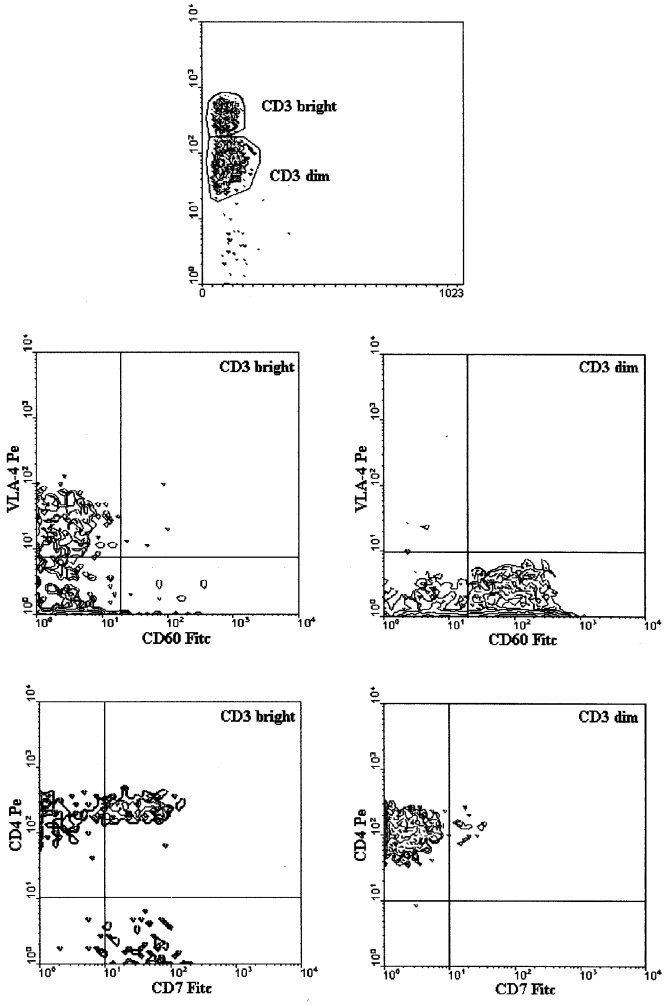

Figure 2. Three-color flow cytometric analysis of, respectively, CD3/CD60/VLA4 and CD3/CD4/CD7 combination in a representative SzS patient. Live lymphocytes were gated on forward and side light scatter. A bimodal expression of CD3 was observed. $CD3^{bright}$ and $CD3^{dim}$ T cells were gated in order to define their respective phenotypic characteristics. $CD3^{bright}$ cells were mostly $CD60^-$ $VLA4^+$. $CD3^{dim}$ lymphocytes express CD60 but not VLA4 antigen. Within $CD3^{bright}$ cells, $CD4^+$ (and $CD4^-$) cells were mostly $CD7^+$, whereas $CD3^{dim}$ were all $CD4^+$ lymphocytes that had lost CD7 surface antigen. In all cytograms, the lower left quadrants delimit the fluorescence intensities obtained with appropriate isotype and fluorochrome-matched negative control MoAb.

patch/plaque stage of MF constantly outnumbered CD7 negative lymphocytes. By contrast, an expansion of $CD7^-$ T helper lymphocytes occurred both in MF patients in stage III–IV and in SzS cases with an inversion of the $CD7^{+/-}$ ratio of 0.28 and 0.12, respectively.

These findings are in agreement with data from the literature which describe a $CD7^-$ expansion in the majority of patients with CTCL (Haynes *et al*, 1981; Berger *et al*, 1982; Wood *et al*, 1986; Ladastide *et al*, 1990; Harmon *et al*, 1996; Bogen *et al*, 1996). It must be noted, however, that such an increase in these cells may also be found in inflammatory dermatoses (Moll *et al*, 1994) or in the aging process (Kukel *et al*, 1994). For these reasons, we studied the expression of many other surface antigens. We demonstrated that the presence of cells lacking CD7 was directly linked both to an increased percentage and absolute number of several activation/differentiation antigens such as CD60 and CD45R0 and to a decreased expression of other molecules such as CD26 and CD45RA (**Tables III** and **IV**, **Fig 2**). Increased expression of the CD60 antigen in patients with CTCL was already described by Hansen *et al* (1993) but no correlation had been made with $CD7^-$ cells. As it is known that $CD7^-$ cells respond preferentially to several stimuli such as CD2, CD28, or CD60 MoAb, but not to anti-CD3 (Carrera *et al*, 1988; Hansen *et al*, 1993), then the increased number of $CD7^-$ $CD60^+$ cells we observed might have a functional significance in these patients. In fact, *in vitro* challenge of T cells from patients with CTCL with CD60, an antigen independent activating molecule, results in frank activation of these cells suggesting that this activation pathway may also be important *in vivo* (Hansen *et al*, 1993). Furthermore, the increased expression of CD45R0 surface antigen in these cells suggests that an expansion of memory cells occurs in the late phases of MF and SzS, as both CD45R0 and CD60 are coexpressed in those cells which switch from a naive to a memory phenotype (Akbar *et al*, 1988; Kniep *et al*, 1996).

Surprisingly, we found decreased expression of CD26 in circulating memory $CD4^+$ lymphocytes in the late phase of CTCL and in SzS cases (**Table III**). $CD4^+CD7^-$ lymphocytes normally express CD26, an important accessory molecule for T cell activation (Reinhold *et al*, 1996). We do not currently know what the significance of this downregulation is, but it is known that there is decreased responsiveness to mitogenic stimuli seen in PBL from patients with SzS (McCusker *et al*, 1997). As CD26 expression is strictly related to protein kinase C activity (Hollsberg *et al*, 1993), the absence of CD26 found in our series might be related to downregulation of protein kinase C activity and, therefore, may explain the above-mentioned abnormal mitogen responsiveness in patients with SzS. It would be of interest to assess the protein kinase C activity in cell lines from patients with SzS in order to validate this supposition.

Another important characteristic of CTCL leukemic cells is their epidermotropism. Several authors have reported that epidermotropic lymphocytes are primarily CLA^+ T cells expressing CD45R0 (Picker *et al*, 1990; Heald *et al*, 1993) and that the expression of CLA antigen is strictly related to the extent of CTCL disease (Borowitz *et al*, 1993). In this study we also show that cells expressing CLA complex and CD15s are expanded in the late phase of disease and that these antigens are coexpressed (**Fig 1**). This indicates that in PBL of patients with CTCL, an increase of skin committed memory T lymphocytes takes place. An interesting finding was the lack of VLA4 (CD49d) expression in PBL of patients with stage III–IV MF and patients with SzS (**Table III**) in the presence of an unmodified absolute number of $VLA4^+$ cells. On the contrary, VLA4 is highly expressed on T lymphocytes that infiltrate the skin (Savoia *et al*, 1992). This difference might reflect the fact that our study was performed on circulating T cells, and did not address the phenotype of the same cells in lymph nodes or skin, where lymphocytes may display different markers. Another explanation of this phenomenon, however, could be that $CD7^-$ circulating T cells, which lack the VLA4 antigen, might represent the counterpart of skin infiltrating lymphocytes that have lost their epidermotropic properties. In fact, VLA4 is thought to exist in multiple activation states (Woldemar Carr *et al*, 1996; Newhan *et al*, 1998): inactive, partially active, and fully active. In the inactive state, this integrin is unable to interact with VCAM-1 or fibronectin. In the partially active state, it binds VCAM-1 but not fibronectin. In the fully activated state, VLA4 is able to bind both ligands. Consequently, the absence of VLA4 on cells expressing CD15s may lead us to hypothesize that CD7 negative T cells in peripheral blood of patients with late-stage disease, although committed to the skin, are unable to migrate through the vessel wall and firmly adhere to the extracellular matrix spaces because of the defective expression of VLA4. The above-mentioned downregulation of CD26, a molecule which may also interact with extracellular matrix proteins (Kameoka *et al*, 1993), adds more efforts to this scenario. Nevertheless, further functional experimental studies are required in order to confirm this hypothesis.

Findings observed in patients with SzS deserve a separate debate. In those patients who are characterized by a large amount of circulating malignant cells, the aberrant distribution of all described antigens may be strictly related to a direct measurement of neoplastic cells. All patients studied with SzS showed two circulating T cell subsets distinguished by phenotype (bright and dim bimodal CD3 expression) as previously described (Bogen *et al*, 1996) or physical (cell size) parameters. Whereas no difference in antigen expression was observed in $CD3^{bright}$ or smaller-sized cells from normal donors, $CD3^{dim}$ or the large-sized lymphoblast subset showed a $CD60^+CD45R0^+$ phenotype associated with the lack of expression of CD7 and CD49d surface antigens (**Fig 2**). Interestingly, patients with MF in late phases of the disease showed similar phenotypical features suggesting these populations might constitute a typical expansion in patients with CTCL with a more aggressive characteristic. Of course, it would be of interest to evaluate whether these cells represent a malignant or reactive population. The correlation of serial determinations of these markers and tumor burden would also be of interest.

As widely reported, an increment of $CD3^+CD4^+$ cells could be observed especially in patients with MF stage III–IV and SzS. $CD3^+CD8^+$ cells, however, tended to diminish from MF stage I–II through MF stage III–IV. These data support the evidence that a reduced tumor-specific cytotoxic activity might occur in CTCL disease (Seo *et al*, 1998) and therefore might be responsible for tumor progression from the earliest phases of disease. Whereas this percentage decrement is also observed in patients with SzS, no difference was noted in the absolute number. This might suggest the decrement of the CD8 compartment is a paraneoplastic phenomenon. Alternatively, the preserved absolute number of CD8 cells may represent a reactive phenomenon vs. leukemic cells.

In conclusion, the flow cytometry panel used in this study allowed us to define a cell population of PBL in patients with CTCL with interesting hallmarks of activation and skin homing ($CD3^+CD4^+CD7^-CD15s^+CD26^-CD45RO^+CD49d^-CD60^+$). This cell population was clearly seen in patients with stage III–IV MF as well as in individuals with SzS where it was also associated with peculiar morphologic and phenotypical parameters (large size, $CD3^{dim}$). The further characterization of this cell population through clonality assessment and the correlation to disease progression through serial analysis, comparing these tests with a more sensitive severity score, will give clues on the functional and pathologic implications of these cells.

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