Utility of flow cytometry immunophenotyping and DNA ploidy studies for diagnosis and characterization of blood involvement in CD4⁺ Sézary's syndrome

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BACKGROUND AND OBJECTIVES. The exact immunophenotypic criteria for the identification of Sézary cells in the blood are still poorly defined.

DESIGN AND METHODS. We analyzed the immunophenotype and DNA content cell of blood T cells in a series of 18 consecutive cases of Sézary's syndrome (SS), 21 normal individuals and 10 patients with reactive erythroderma, and correlated them with molecular and morphological findings.

RESULTS. Phenotypically abnormal CD3⁺/TCRαβ⁺/CD4⁺ T cells were found in all SS patients but in none of the reactive erythroderma cases; small diploid, or less frequently hypodiploid Sézary's cells coexisted with large nearly tetraploid Sézary's cells in some cases. The most frequent phenotypic aberrations consisted in decreased expression of CD3/TCRαβ (94%), CD4 (94%), CD7 (100%) and/or CD2 (83%). In addition, Sézary's cells were constantly CD28⁺ and CD5⁺ and they did not express natural-killer associated (Nka) antigens. Phenotypic heterogeneity was a common finding and phenotypic changes over time were frequently observed. In contrast to what was found in patients with reactive erythroderma, flow cytometry analysis of the T-cell receptor (TCR) repertoire revealed a major TCR-Vβ expansion in all SS cases.

INTERPRETATION AND CONCLUSIONS. The presence of CD28⁺/CD5⁺/Nka⁺/CD4⁺ T cells expressing abnormally low levels of CD3, TCRαβ, CD4, CD7 and/or CD2 would support the diagnosis of SS in patients with erythroderma. Further analyses on larger series of patients are necessary in order to cover less frequent phenotypic patterns, establish the preferential usage of specific TCR-Vβ families and investigate the specificity of these phenotypic abnormalities for diagnosing SS.

Key words: erythroderma, Sézary's syndrome, cutaneous T-cell lymphoma, flow cytometry.

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Sézary's syndrome (SS) is a rare form of cutaneous T-cell lymphoma (CTCL) originally defined by the co-existence of erythroderma, lymphadenopathy and > 10% atypical mononuclear cells – Sézary's cells – in the blood. Most SS patients are elderly males and the syndrome may arise de novo or from pre-existing lesions histopathologically classified as mycosis fungoides (MF). The clinical presentation is a non-specific erythroderma which may be accompanied by pruritus, fever, chills, weight loss and malaise; scaling, fissuring of palms and soles; alopecia, ectropion, nail dystrophy and peripheral lymphadenopathy may also occur.

Previous studies have shown that Sézary T cells express the cutaneous lymphocyte-associated antigen, a skin-selective homing receptor,1,2 have a particular affinity to lesional keratinocytes3,4 and mainly transcribe the interleukin-10 and interleukin-5 T-helper 2 cytokines.5,6 These properties may explain, at least to a certain extent, the cutaneous tropism and the immunological abnormalities found in SS, which include both elevated serum IgE levels and eosinophilia.

Although erythroderma is the clinical hallmark of SS, it may also occur in a large range of reactive and malignant conditions, such as atopic dermatitis, chronic actinic dermatitis, psoriasis and drug reactions.7 In addition, erythroderma may result from skin infiltration by other neoplastic hematologic cells.8 Thus, detection of both skin infiltration, consisting of a perivascular accumulation of atypical CD4⁺ T cells with convoluted (cerebriform) nuclei in the dermis with epidermotropism, and the presence of Sézary cells in blood have been considered necessary factors for establishing a diagnosis. Nevertheless, non-specific cutaneous histopathologic findings are frequent in SS and multiple skin biopsies may be needed because of marked variability of the infiltrate density and epidermotropism, the latter usually less evident than in MF.9,10 Difficulties in morphologically evaluating the presence of Sézary cells in blood might also occur, since atypical lymphocytes can be found in patients with reactive forms of erythroderma and as a consequence, the Sézary cells may represent only a minor fraction of all blood lymphocytes in SS. Accordingly, the diagnosis of SS based on clinical–pathologic grounds alone is not always straightforward, particularly in the early stages of the disease, and it is now well accepted that additional criteria are needed.11

Several studies have shown that Sézary cells frequently display aberrant phenotypes including abnormal expression of CD7,11-16 CD3,15,17 and CD2.15,18,19 Despite this, some
authors claim that the presence of clonal T cells in the blood should be confirmed by molecular studies.20 Nevertheless, molecular studies also have limitations that compromise their use in routine diagnosis and do not provide helpful information to further sub-classify the disease.21-22

Based on the results of the present study, involving a comparative analysis of 18 cases of SS, 10 patients with reactive erythroderma and 21 normal individuals, we propose that the presence of a TCR-αβ-restricted CD5+, CD28+, NK-associated antigen (NKa)+, CD4+ T-cell population with a CD3+/low, TCR+low, CD4+/low, CD2+/dim, CD7+/low phenotype in the blood will make an enormous contribution to the routine diagnosis of SS in patients presenting with erythroderma.

Design and Methods

Patients and samples

A total of 18 consecutive patients with SS (13 males and 5 females, with a median age of 73 years, range 53 to 87 years) were studied at diagnosis. None of these patients had a past history of MF; although six of them (patients #5, 6, 10, 13, 14, 15 and 17) had had extended periods (from 1 to 9 years) of erythroderma that had been mis-diagnosed as reactive dermatitis based on clinical grounds. Clinical features observed at diagnosis were those typically found in this condition and included erythroderma in all cases, skin nodules in 1 patient (patient #5) and lymphadenopathy in 8 patients (patients #3, 5, 7, 11, 12, 15, 17 and 18). Morphologic analysis of blood smears showed the presence of atypical lymphocytes compatible with Sézary cells in 15 cases. Skin biopsy, bone marrow aspirate and biopsy and lymph node biopsy were consistent with SS. Long-term follow-up studies (mean follow-up of 21 months from diagnosis; range: 18 to 35 months) were performed in 4 cases.

In addition, 10 patients with reactive erythroderma including photodermatitis (n = 3), psoriasis (n = 2), toxicodermia (n = 1), atopic dermatitis (n = 1), chronic eosinophilic leukemia (n = 1), and erythroderma of unknown origin (n = 2), were studied. These ten patients (7 males and 3 females) had a median age of 51 years (range: 23 to 81 years).

Normal reference values were based on the study of blood samples from 21 normal healthy adults (age range: 18 to 51 years).

Blood analyses included in this study were: (i) flow cytometric lymphocyte immunophenotyping, (ii) immunophenotypic assessment of the TCR-β chain variable (TCR-β) region repertoire, (iii) determination of DNA cell content, and (iv) molecular analysis of TCR-β and -γ gene rearrangements. All these studies were performed in blood samples collected in tripotassium EDTA tubes.

Immunophenotypic studies

Flow cytometric immunophenotypic studies were performed on whole blood, using a stain-lyse-and-wash method (FACS lysing solution, Becton/Dickinson Biosciences – DBB – San José, CA, USA) and 4-color stainings that combined fluorescein isothiocyanate- (FITC), phycoerythrin- (PE), peridinin chlorophyll protein- (PerCP) or PE-cyanine 5- (PC5) and allophycocyanine (APC) –conjugated monoclonal antibodies (MAb). In a first step, NK and T cells as well as the major TCRαβ+, TCRγδ+, CD4+ and CD8+ T-lymphocyte subsets were quantified using the following combinations of MAb (FITC/PE/PerCP or PC5/APC): CD8/CD28/CD4/CD3, TCRαβ/CD8/CD4/CD3, CD2/CD7/CD56/CD3 and CD57/CD11c/CD56/CD3. In a second step, CD4+/TCRαβ+ and CD8+/high/TCRαβ+ T-cell subsets were further characterized, by combining APC-conjugated anti-CD8 and PerCP-conjugated anti-CD4 with a large panel of FITC/PE-conjugated MAb directed against different T- and NK-cell associated antigens as well as lymphoid activation markers and adhesion molecules: CD2/CD7, CD7/CD5, CD11a/NL-ΔR, CD122/CD25, CD45RA/CD45RO, CD11c/CD57, CD16/CD56. In addition, the expression of CD62L (FITC), CD94 (FITC), CD158a (FITC), CD161 (PE) and NKb1 (PE) was investigated in a total of 8, 6, 7, 11 and 11 SS patients, respectively. Whenever necessary, different combinations of the above listed MAb were used in order to characterize the neoplastic T cells better.

A similar strategy combining APC-conjugated anti-CD8 and PerCP-conjugated anti-CD4 with FITC/PE-conjugated anti-TCR-β MAb was used for the analysis of the TCR-β repertoire. For this purpose, a total of 24 MAb directed against an identical number of members of 19 different TCR-β families (TCR-β1.1, -Vβ2.1, -Vβ3.1, -Vβ5.1, -Vβ5.2, -Vβ5.3, -Vβ6.1, -Vβ6.7, -Vβ7.1, -Vβ8.1, -Vβ8.2, -Vβ9.1, -Vβ11.1, -Vβ12.2, -Vβ13.1, -Vβ13.6, -Vβ14.1, -Vβ16.1, -Vβ17.1, -Vβ18.1, -Vβ20.1, -Vβ21.3, -Vβ22.1 and -Vβ23.1) (Immunotech, Marseille, France) were used. This panel covers about 60±4% and 46±6% of the TCR-β repertoire of the CD4+ and CD8+high T-cells present in normal blood, respectively.24 Criteria used to define a TCR-β expansion were previously defined in detail.24 Briefly, for those TCR-β families that were assessed with the panel of MAb used in this study (direct identification), a TCR-β expansion was considered to be present whenever its representation exceeded by at least two standard deviations (SD) the mean value observed in CD4+ or CD8+high T-cells in normal healthy individuals. For the remaining TCR-β families that were not explored with the panel of MAb used, the criteria used to define a TCR-β expansion (indirect identification) was based on the observation of a relative decrease in the fraction of either CD4+ or
CD8^bright circulating T cells that were recognized with the panel of MAbs to values < 85% of those observed for the same T-cell subsets in blood samples from normal adult individuals. Whenever necessary, additional appropriate multicolor stainings were used in order to confirm whether a given TCR-ββ expansion had occurred within either the lymphomatous or normal T-cell compartments.

Data acquisition was carried out in a FACSCaliburTM flow cytometer equipped with a 488 nm argon laser and a 625 nm neon diode laser (BDB). Paint-A-Gate PRO software (BDB) was used for data analysis. First, CD4^+ T cells were gated and analyzed for the presence of phenotypically abnormal and/or expanded T-cell populations. Once identified, the abnormal T cells were analyzed for each antigenic determinant tested. The following parameters were recorded for each marker: (i) percentage of positive cells; (ii) intensity of expression evaluated as the mean fluorescence intensity (MFI); (iii) pattern of expression – homogeneous versus heterogeneous – evaluated by the coefficient of variation (CV). In addition, the light scatter properties and the intensity of expression of each antigen on the phenotypically aberrant CD4^+ T cells was compared to that observed in the phenotypically normal CD4^+ T cells by calculating the ratio between the MFI values obtained for each antigen in these cell populations (fold increase or fold decrease). Abnormal CD4^+ T cells were considered to be increased in size (large) when the ratio between their forward light scatter (FSC) and the FSC of the normal residual CD4^+ T cells was higher than 1.5.

CD4^+ and CD8^+ cytotoxic T cells (CTL) were defined by flow cytometry as CD28^- T cells cells with high FSC and sideward light scatter (SSC) properties and expressing one or more natural NKa markers from those tested.

**Flow cytometric DNA cell content studies**

Flow cytometric DNA cell content studies were performed in whole blood, using a double staining technique for surface CD4 and nuclear DNA, previously described in detail. Briefly, cells were stained with FITC-conjugated mouse anti-human CD4, washed twice, and then stained with a secondary FITC-conjugated rat anti-mouse immunoglobulins reagent (DAKO A/S, Glostrup, Denmark); afterwards, cells were washed once and treated with DNA-Prep reagents (Beckman/Coulter – B/C–, Hialeah, FL, USA) according to the manufacturer’s instructions. Other stainings (CD2/DNA, CD3/DNA and CD7/DNA) were also performed whenever necessary to distinguish between normal and abnormal CD4^+ T cells.

Data acquisition was carried out in an EPICS-XL-MCL flow cytometer (B/C) equipped with a 15-mW air-cooled 488 nm argon laser, using the XL2 software program (B/C). Analysis of the DNA content and calculation of both the DNA ploidy and the cell cycle distribution of the neoplastic T-cell population was performed with MultiCycle software (Phoenix Flow Systems, San Diego, CA, USA). The DNA index was calculated by dividing the modal DNA cell content of the abnormal CD4^+ T-cell population by that of the normal residual cells present in the same sample.

**Molecular analysis of T-cell receptor gene rearrangements**

Molecular analysis of TCR gene rearrangements was performed using conventional Southern blot techniques as previously described in detail. Briefly, mononuclear cells were obtained by density gradient centrifugation on Lymphoprep® (Nycomed Pharma AS, Oslo, Norway), washed twice in phosphate-buffered saline (PBS) and cryopreserved. DNA was extracted using the chloroform method and digested with EcoRI and HindIII restriction enzymes. DNA fragments were separated by a 0.8% agarose gel electrophoresis and transferred to nitrocellulose membranes by vacuum blotting, UV fixed and hybridized with 32P-labeled probes for the TCR-β gene region (Cβ, TCRBC and TCRBJ2; DAKO A/S). Clonality studies were also performed by PCR analysis of TCR-β gene (n= 6) and/or TCR-β gene (n= 7) rearrangements, using the strategies and primers previously described.

**Statistical analysis**

For all variables under study, median, mean, standard deviation and range values were calculated. Comparisons between groups were made using the Mann–Whitney U test (SPSS 11, SPSS, Chicago, IL, USA). The degree of linear correlation between variables was evaluated by calculating Pearson’s product-moment correlation coefficient (CoStat version 6.102, CoHort Software, CA, USA). χ^2 values were also computed for further assessment of the overall concordance between the incidences of specific TCR-ββ family members with their representation in normal samples, using the SPSS software.

**Results**

**Distribution of the major T-cell subsets in the blood of patients with SS at diagnosis**

Patients with SS showed highly variable white blood cell (WBC) and lymphocyte counts (Table 1), although the absolute number of CD4^+ T-cells was increased (> 1100×10^6/L) in all but 2 cases (mean of 5490±4991×10^6/L; range: 832×10^6/L to 15710×10^6/L), leading to an increased CD4/CD8 ratio in most patients (mean: 38.4±59.6) (see also Table 1).

In all cases, phenotypically abnormal TCRαβ+/ CD4^+ T-cells were identified in blood. The abnormal
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CD4+ T-cell population accounted for between 14 and 99% (mean: 79±26%; median: 89%) of all CD4+ T cells.

In 10 cases (patients #1-4, 11, 13-16 and 18), only small Sézary cells with similar light scatter characteristics to those of normal residual CD4+ T cells (mean FSC fold increase of 1.0±0.1, range: 0.9 to 1.3) were found. In contrast, in the remaining 8 cases (patients 5-10, 12 and 17), small Sézary cells co-existed with a large sized CD4+ Sézary T-cell population (mean FSC fold increase of 1.8±0.2, ranging from 1.6 to 2.1), these latter cells accounting for most (median value of 96.1%; range: 53.6 to 99.9%) Sézary cells in the blood (Table 1). Four out of the 8 patients with large Sézary cells (50%) had a long past history (> 1 year) of erythroderma, as compared to 3 out of the 10 patients in whom only small Sézary cells were found (30%).

Phenotypically abnormal CD4+/TCRαβ+ T-cells co-existed with variable numbers of both normal residual CD4+/TCRαβ+ (453±374×10^6/L) and CD8+/TCRαβ+ T-cells (387±421×10^6/L, most cases (n=15) showing decreased numbers of both normal CD4+/TCRαβ+ (< 700×10^6/L) and CD8+/TCRαβ+ (< 500×10^6/L) T lymphocytes.

**Immunophenotypic features and DNA cell contents of CD4+/TCRαβ+ Sézary cells at diagnosis**

Figure 1 illustrates the typical immunophenotypic patterns observed in Sézary cells, concerning the antigens analyzed here and Figure 2 shows the most relevant phenotypic aberrations found on CD4+ neoplastic cells as compared to normal residual CD4+ T cells. As may be seen in this latter figure, CD3, TCRαβ and CD4 expression were decreased in all but one case (patient 12), the MFI obtained for these antigens being decreased by 1.2 to 8.7 (median: 2.1), 2.2 to 10.1 (median: 3.8) and 1.1 to 4.3 fold (median: 1.8), respectively. In contrast to what was

### Table 1. Blood cell counts and major phenotypic data (number total CD4+ T-cells, the CD4/CD8 ratio, the representation of the phenotypically abnormal CD4+ T-cell population and the TCR-Vβ expansions found on CD4+ T-cells in the blood) in patients with Sézary’s syndrome (n=18).

<table>
<thead>
<tr>
<th>Patient</th>
<th>WBC (×10^9/L)</th>
<th>Lymphocytes from WBC</th>
<th>% Hb</th>
<th>Platelets (×10^9/L)</th>
<th>% CD4+ T-cells from total lymphocytes</th>
<th>CD4/CD8 ratio</th>
<th>% abnormal CD4+ T-cells from total CD4+ T-cells</th>
<th>% expanded TCR-Vβ family from total CD4+ T-cells</th>
<th>% large abnormal CD4+ T-cells from total CD4+ T-cells</th>
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<tbody>
<tr>
<td>1</td>
<td>5.1</td>
<td>49.0</td>
<td>15.6</td>
<td>158</td>
<td>57.5</td>
<td>2.7</td>
<td>38.6 (10.9)</td>
<td>Vβ13.6 – 48%</td>
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</tr>
<tr>
<td>2</td>
<td>15.5</td>
<td>40.8</td>
<td>13.6</td>
<td>299</td>
<td>82.8</td>
<td>14.8</td>
<td>89.6 (30.3)</td>
<td>Vβ13.6 – 86%</td>
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<tr>
<td>3</td>
<td>25.3</td>
<td>58.2</td>
<td>14.0</td>
<td>203</td>
<td>96.1</td>
<td>199.0</td>
<td>95.6 (53.6)</td>
<td>Vβ13.6 – 97%</td>
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</tr>
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<td>4</td>
<td>25.9</td>
<td>64.2</td>
<td>12.9</td>
<td>251</td>
<td>94.5</td>
<td>162.0</td>
<td>98.1 (59.5)</td>
<td>Vβ13.6 – 87%</td>
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</tr>
<tr>
<td>5</td>
<td>9.8</td>
<td>15.5</td>
<td>11.5</td>
<td>329</td>
<td>54.7</td>
<td>4.1</td>
<td>61.2 (5.2)</td>
<td>Vβ13.6 – 22%</td>
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<td>6</td>
<td>11.7</td>
<td>61.6</td>
<td>9.9</td>
<td>116</td>
<td>85.8</td>
<td>16.4</td>
<td>98.1 (51.8)</td>
<td>Vβ13.6 – 97%</td>
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<td>7</td>
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<td>12.9</td>
<td>10.6</td>
<td>187</td>
<td>74.2</td>
<td>8.2</td>
<td>45.5 (4.4)</td>
<td>Vβ13.6 – 59%</td>
<td>91.5</td>
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<tr>
<td>8</td>
<td>12.8</td>
<td>18.7</td>
<td>15.4</td>
<td>336</td>
<td>60.7</td>
<td>3.5</td>
<td>73.4 (8.3)</td>
<td>Vβ13.6 – 65%</td>
<td>95.9</td>
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<tr>
<td>9</td>
<td>8.7</td>
<td>39.1</td>
<td>12.8</td>
<td>202</td>
<td>80.6</td>
<td>8.0</td>
<td>87.4 (27.5)</td>
<td>Vβ13.6 – 88%</td>
<td>96.3</td>
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<td>12.3</td>
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<td>81.3</td>
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<td>97.8 (31.5)</td>
<td>Vβ13.6 – 94%</td>
<td>53.6</td>
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<td>16.0</td>
<td>59.1</td>
<td>15.0</td>
<td>117</td>
<td>66.7</td>
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<td>86.9 (34.2)</td>
<td>Vβ13.6 – 75%</td>
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<td>20.8</td>
<td>58.3</td>
<td>8.2</td>
<td>164</td>
<td>92.0</td>
<td>25.2</td>
<td>98.9 (53.0)</td>
<td>Vβ13.6 – 97%</td>
<td>99.8</td>
</tr>
<tr>
<td>13</td>
<td>7.9</td>
<td>55.6</td>
<td>13.5</td>
<td>194</td>
<td>91.2</td>
<td>123.9</td>
<td>96.9 (49.4)</td>
<td>Vβ13.6 – 95%</td>
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<td>14</td>
<td>8.2</td>
<td>17.2</td>
<td>11.2</td>
<td>277</td>
<td>66.1</td>
<td>3.6</td>
<td>51.7 (5.9)</td>
<td>Vβ13.6 – 50%</td>
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<td>23.7</td>
<td>53.6</td>
<td>13.1</td>
<td>205</td>
<td>91.0</td>
<td>19.8</td>
<td>96.0 (46.8)</td>
<td>Vβ13.6 – 91%</td>
<td>0.0</td>
</tr>
<tr>
<td>16</td>
<td>6.7</td>
<td>28.9</td>
<td>12.9</td>
<td>89</td>
<td>83.7</td>
<td>42.2</td>
<td>87.1 (21.1)</td>
<td>Vβ13.6 – 73%</td>
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<tr>
<td>17</td>
<td>17.4</td>
<td>18.7</td>
<td>14.7</td>
<td>298</td>
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<td>3.6</td>
<td>94.9 (11.3)</td>
<td>Vβ13.6 – 87%</td>
<td>99.9</td>
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<tr>
<td>18</td>
<td>10.5</td>
<td>32.1</td>
<td>13.0</td>
<td>250</td>
<td>55.1</td>
<td>3.7</td>
<td>14.0 (2.5) [95.0 (45.9)*]</td>
<td>Vβ13.6 – 18% (82%)</td>
<td>0.0</td>
</tr>
<tr>
<td>Mean±SD</td>
<td>14.0±6.3</td>
<td>37.7±16.9</td>
<td>13.0±2.2</td>
<td>215±70</td>
<td>76.6±14.4</td>
<td>38.4±59.6</td>
<td>78.4±25.4</td>
<td>72.6±26.8</td>
<td>40.1±47.2</td>
</tr>
</tbody>
</table>

TCR, T-cell receptor; Vβni, the expanded TCR-Vβ family was not positively identified with the panel of MAbs used in this study. *Values in brackets were found 25 months after diagnosis.
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observed in normal blood samples — in which CD3 levels on CD4+/TCRαβ+ T cells always exceeded those on CD8+/TCRαβ+ T cells (fold increase ranging from 1.1 to 1.3) — CD3 levels on CD4+/TCRαβ+ Sézary T-cells were lower than those observed on CD8+/TCRαβ+ T-cells present in the same sample in 12 out of 18 cases (fold decrease ranging from 1.1 to 10.2).

In addition, Sézary cells also showed decreased CD7 expression, with the antigen being either absent (n=9) or dimly expressed (n=8; fold decrease: 1.1 to 5.6; median: 2.0) (Figure 2); a bimodal CD7 expression was noted in half of patients (n=9/18) due to the co-existence of both CD7- and CD7+ Sézary cells. Similarly, abnormally low CD2 expression was found in 16 out of 18 cases studied (Figure 2), the marker being either negative (n=1) or expressed with a decreased intensity (n=15; fold decrease of 1.2 to 14.1 with a median value of 2.4) as compared to normal residual CD4+ T cells. Combined staining for CD2 and CD7 was of a great help in distinguishing normal activated CD7+low CD4+ T-cells, which express CD2high (CD2high/CD7+/low) from CD7+/low CD4+ Sézary T-cells, which expressed abnormally low CD2 levels (Figure 1). Overall, 5 cases showed a multimodal CD2 expression, due to the coexistence of CD2- and one or two different subpopulations of CD2+ in Sézary cells.

In contrast to expression of CD7 and CD2, Sézary cells were constantly CD5+high, although CD5 expression was rather variable, being either lower (n=9; median decrease of 1.5 fold, ranging from 1.1 to 2.6) or higher (n=9; median increase of 2.2 fold, ranging from 1.3 to 4.7; n=9) than that of normal residual CD4+ T cells. Like CD5, CD28 was constantly expressed on Sézary T cells, with its intensity of expression increased in half of the cases (median increase of 1.7; range: 1.2 to 2.2), normal in 2 cases and decreased by 1.1 to 3.2 folds (median of 1.3) in the remaining 7 patients. Like recently activated T cells, in most cases (n=13) CD4+ Sézary cells were CD45RO+/CD45RA-, the other 5 patients showing co-expression of CD45RO and CD45RA. In addition, CD4+ Sézary cells showed variable expression of CD62L and CD25, with both markers dimly expressed in half of the cases tested, and they were CD122-. However, in contrast to normal recently activated T cells, which usually display a CD11a+/high/CD38+/HLA-DR+ phenotype, CD4+ Sézary cells constantly showed a CD11a+/low/CD38+/HLA-DR+/low phenotype (mean percentage of HLA-DR+ cells: 11±10%). Dim co-expression of CD8 on 50% of CD4+ Sézary cells was observed in one case. The CD11b, CD11c, CD16, CD56, CD94, CD158a, and NKB1 natural killer -associated molecules and killer-receptors were negative in all except two cases, one (case #6) displaying a very dim and heterogeneous expression of CD56 and the other (case #9) being CD158a+low. Comparative immunophenotypic analysis of the small and large Sézary cell populations was performed in only one case (patient #10, in whom small and large Sézary cells accounted for 46% and 54% of the total Sézary cells, respectively). In this specific case, small and large Sézary cells differed mainly in the levels of their expression of CD2 and CD7 molecules.

In fact, small Sézary cells were CD7+ and CD2-/+low, whereas large Sézary cells were CD7+/low and expressed higher levels of CD2. Moreover, the intensity of expression of CD4, CD5, CD11a and CD25 and the percentage of HLA-DR+ cells (0% versus 30%) was higher whereas that of CD3/TCRαβ was lower on large Sézary cells. In the other cases in which both small and large Sézary cells were identified, the representation of the small-sized Sézary cell population (5±5% of total Sézary cells) did not
Blood involvement in Sézary syndrome

allow an accurate comparison of the immuno-
phenotypic features of small and large Sézary cells.

TCR-Vβ repertoire studies of CD4+/TCRαβ+ revealed a TCR-Vβ expansion in all cases, accounting for 18 to 97% of circulating CD4+ T cells (mean: 73±27%; median: 86%). The TCR-Vβ restricted CD4+ T-cell population corresponded to the phenotypically abnormal T-cell population, as reflected by the high correlation between the magnitude of the TCR-Vβ expansion and the fraction of phenotypically abnormal CD4+ T cells observed in each individual case (r=0.896; p<0.0001) (Figure 3). The expanded TCR-Vβ family was not identified in the majority (14/18) of cases; in the 4 cases in which the expanded TCR-Vβ family was identified, it corresponded either to TCR-Vβ13.6 (n=3) or to TCR-Vβ2.1 (n=1) (Table 1). Taking into account that the panel of anti-TCR-Vβ MAb used allowed us to identify 60±4% (ranging from 53 to 66%) of the TCR-Vβ repertoire of the normal blood CD4+ T cells,24 the frequency of

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**Figure 2. Aberrant expression of T-cell associated markers on CD4+ Sézary T-cells.** Results are expressed as fold variation (increase or decrease) on the mean fluorescence intensity of each individual antigen on CD4+ Sézary T cells as compared to the normal residual CD4+ T cells from the same sample. Absence of antigen expression is shown as white bars.

**Figure 3. Correlation between the fraction of phenotypically abnormal CD4+ T cells and the TCR-Vβ restricted clonal expansions among total CD4+ T cells in the blood of SS patients.**
cases in which we were able to identify the expanded TCR-Vβ family was significantly lower than that expected if no restricted usage of specific TCR-Vβ families occurred in neoplastic cells from SS patients (p=0.01).

Molecular studies confirmed the existence of monoclonal rearrangements of the TCR β-chain gene in all patients with SS. No evidence of the existence of multiple TCR-β or TCR-γ gene rearrangements were found in any of the 18 SS cases analyzed.

Flow cytometric analysis of the DNA contents of abnormal CD4+ T cells performed in 5 cases in which only small Sézary cells were identified, showed either diploid (n=3; DNA index of 1.01±0.01, ranging from 1.01 to 1.02) or hypoploid DNA content (n=2; DNA index of 0.89 and 0.94). In contrast, all 3 cases analyzed in which large Sézary cells were found, showed one (n=2) or more (n=1) DNA hyperdiploid CD4+ T-cell populations (DNA index of 1.83±0.15; range: 1.60 to 1.99). In these latter cases, hyperdiploid cells constantly corresponded to large neoplastic CD4+ T cells and co-existed with a small proportion of small Sézary cells with a diploid DNA content (DNA index of 1.00±0.03). In terms of the cell cycle distribution, the percentage of S phase cells was 1.1±0.7% and 0.7±1.1% and the percentage of G2/M phase cells, 0.4±0.3% and 1.1±2.3%, for small and large Sézary cells, respectively.

**Immunophenotypic changes of CD4+/TCRααββ Sézary cells during follow-up**

Long-term follow-up complete immunophenotypic studies (mean follow-up of 21 months; range: 18 to 35 months) were available in 4 patients with SS. Phenotypic changes occurred over time in all these cases and they mainly involved CD2 (n=3) and CD7 (n=3) expression. Differences were found between patients. In case #18 (Figure 4, panel A), who had the lowest proportion of Sézary cells in the blood at diagnosis, a progressive decrease in CD2 expression on the Sézary cells associated with up-regulation of CD25 was observed, with the overall proportion of phenotypically normal TCR-Vβ-restricted Sézary cells also increasing in parallel in blood. In another case, patient #10 (Figure 4, panel B), in whom both small and large Sézary cells were identified at diagnosis, the number of large Sézary cells decreased selectively after treatment and increased thereafter as disease progressed again. Simultaneously, in this patient there was a progressive increase in CD2 expression on small Sézary cells and a progressive decrease in CD7 expression on large Sézary cells. Phenotypic changes observed in two additional cases consist-

Figure 4. Light scatter and CD2/CD7 dot plots illustrating the phenotypic changes that occurred over time in two patients: patient 18 (panel A) and patient 10 (panel B). Normal residual CD4+ T cells are shown in black, small CD4+ Sézary cells are shown in red and large Sézary cells are shown in green. Values inside dot plots indicate the percentage of each lymphocyte population among the total WBC. Dot-plots shown in the upper row were obtained at diagnosis whereas those in the middle and lower rows were obtained at different times during disease evolution.
ed in a progressive increase in the fraction of large CD4+ Sézary cells together with a decrease in CD7 expression in one case and a decrease in CD2 expression on Sézary cells in the other case.

### Immunophenotypic features of normal residual T cells in the blood of patients with SS at diagnosis

Comparing the immunophenotypic features of normal residual CD8+/TCRβ+ T cells in the blood of patients with SS and those of normal individuals, an increased fraction of CD2+high/CD7-/+low (88 ± 20% vs. 49±11%, p< 0.001), CD11a+high (72±32% vs. 46±12%, p=0.007) and HLA-DR+ (51±19% vs. 15±6%, p<0.001) was found. In addition, SS patients also showed an increased fraction of CD45RA+CD45RO+ (30±9% vs. 17±14%; p=0.01) CD4+/TCRβ+ T cells.

In addition, among normal residual CD4+ T-cells from patients with SS, an increased fraction of cells with a CTL-associated immunophenotype was observed, as reflected by the mean percentage of CD28+ (55±28% vs. 30±8%; p=0.02) CTL-association phenotype (Table 2).

Normal residual blood CD4+/TCRβ+ T cells from SS patients also showed higher expression of activation-related markers than did CD4+/TCRβ+ T cells from normal individuals, as evaluated by the fraction of CD2+high/CD7-/+low (63±22% vs. 48±10%; p=0.02), HLA-DR+ (31±18% vs. 8±2%; p=0.001) cells; the SS patients also showed a higher proportion of CD45RO+ (83±14% vs. 70±9%; p=0.02) and a lower percentage of CD45RA+/CD45RO- (30±9% vs. 17±14%; p=0.01) CD4+/TCRβ+ T cells.

In contrast to what was observed in SS patients, the absolute number of CD4+ T-cells was increased (> 1100×10⁶/L) in only 4 out of 10 patients with reactive erythroderma (mean of 1010±654 ×10⁶/L; range: 282×10⁶/L to 2421×10⁶/L), leading to an increased CD4/CD8 ratio (CD4/CD8 > 3.9) in only 2 patients (mean of 2.83±1.66; range: 0.68 to 6.36) (Table 3).

The major phenotypic differences between CD4+ T-cells from these patients and those from normal individuals consisted of increased FSC (p<0.01) and SSC (p<0.001), increased CD2 expression (p<0.001) and decreased CD7 (p<0.001) expression (Figure 5). In addition, these cases showed higher percentages

### Table 2. PB CD8+ T-cells in CD4+ Sézary’s syndrome patients: expression of CD28, NK-associated (NKa) antigens (CD56 and/or CD57) and TCR-Vβ expansions.

<table>
<thead>
<tr>
<th>Patient no.*</th>
<th>% CD8+ cells</th>
<th>% NKa+ cells</th>
<th>Expanded TCR-Vβ families</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>ND</td>
<td>ND</td>
<td>Vβ16.1 (45.5%)</td>
</tr>
<tr>
<td>2</td>
<td>89.2</td>
<td>88.5</td>
<td>Vβ21.3 (64.1%)</td>
</tr>
<tr>
<td>3</td>
<td>25.6</td>
<td>38.2</td>
<td>Vβ21.3 (9.3%)</td>
</tr>
<tr>
<td>4</td>
<td>45.1</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>5</td>
<td>90.9</td>
<td>91.6</td>
<td>Vβ5.9 (81.5%)</td>
</tr>
<tr>
<td>6</td>
<td>ND</td>
<td>ND</td>
<td>Vβ16.1 (11.6%)</td>
</tr>
<tr>
<td>7</td>
<td>93.2</td>
<td>94.0</td>
<td>Vβ12.1 (12.1%)</td>
</tr>
<tr>
<td>8</td>
<td>61.6</td>
<td>62.2</td>
<td>Vβ21.3 (0.8%)</td>
</tr>
<tr>
<td>9</td>
<td>ND</td>
<td>ND</td>
<td>NI</td>
</tr>
<tr>
<td>10</td>
<td>43.2</td>
<td>35.7</td>
<td>Vβ20.1 (16.2%)</td>
</tr>
<tr>
<td>11</td>
<td>73.9</td>
<td>30.3</td>
<td>Vβ6 (64.0%)</td>
</tr>
<tr>
<td>12</td>
<td>11.2</td>
<td>0.2</td>
<td>NI</td>
</tr>
<tr>
<td>13</td>
<td>48.7</td>
<td>43.1</td>
<td>Vβ5.1 (10.1%)</td>
</tr>
<tr>
<td>14</td>
<td>9.0</td>
<td>7.9</td>
<td>Vβ14.1 (6.2%)</td>
</tr>
</tbody>
</table>

*Cases 5 and 9 were not analyzed.

Results expressed as percentage from total CD8+ peripheral blood T-cells. NI: No TCR-Vβ expansion identified; Vβ: the expanded TCR-Vβ family was not positively identified with the panel of anti-TCR-Vβ MAb used in this study.

**Distribution of the major T-cell subsets and immunophenotypic features of CD4+/TCRβ+ T cells in the blood of patients with reactive erythroderma**

In contrast to what was observed in SS patients, the absolute number of CD4+ T-cells was increased (> 1100×10⁶/L) in only 4 out of 10 patients with reactive erythroderma (mean of 1010±654 ×10⁶/L; range: 282×10⁶/L to 2421×10⁶/L), leading to an increased CD4/CD8 ratio (CD4/CD8 > 3.9) in only 2 patients (mean of 2.83±1.66; range: 0.68 to 6.36) (Table 3).

The major phenotypic differences between CD4+ T-cells from these patients and those from normal individuals consisted of increased FSC (p<0.01) and SSC (p<0.001), increased CD2 expression (p<0.001) and decreased CD7 (p<0.001) expression (Figure 5). In addition, these cases showed higher percentages
of HLA-DR (42±2.2% vs. 8±2%, p<0.001), CD25+ (72±16% vs. 55±9%, p<0.05), CD45RO+ (82±14% vs. 77±12%, p<0.01) and CD45RO+/CD45RA− (70±11% vs. 53±12%, p<0.01) and a lower proportion of CD45RA+ (31±10% vs. 47±12%, p<0.01) and CD45RO+/CD45RA− (12±7% vs. 27±10%, p<0.01) T cells. There was also a slight, but statistically significant, increase in the proportion of CD28−/CD4+ T cells (7±8% vs. 2±2%; p<0.05), together with a slight increase in the proportion of CD57−/CD4+ T cells (7±6% vs. 3±2%; p<0.01), as compared to in normal individuals. CD94, CD158a and NKB1 expression on CD4+ T cells was not observed, as found in SS patients and in healthy individuals.

In contrast to what was observed in SS patients, overall levels of CD3/TCRβ and CD4 on CD4+ T cells did not differ significantly from those observed on normal blood CD4+ T cells, exceeding by a factor of 1.4±0.6 those observed on CD8+/TCRαβ− T cells present in the same sample. In addition, abnormal CD3+/CD4+ T cells were never found; instead, blood CD4+ T-cells from these patients usually had a CD3+/CD4+/CD2−/CD7+/CD7−/low T-cell activation-related immunophenotype. Analysis of the TCR-Vβ repertoire of CD4+ T cells showed one (2 cases) or more (3 cases), usually small, TCR-Vβ expansions in only 5 patients, which accounted for 4% to 29% of the total CD4+ T cells (mean: 10±9%). Germ-line TCR genes were found in all 10 patients with reactive erythroderma.

**Discussion**

General agreement exists on the urgent need to define more sensitive and specific criteria to identify Sézary cells. In line with this, the International Society for Cutaneous Lymphomas has recently proposed a new algorithm for diagnosis of blood involvement in SS:

1. An absolute count of 1000 Sézary cells/mm³ or more; and
2. A CD4/CD8 ratio ≥ 10 or higher and/or an aberrant loss of expression of pan-T cell markers by flow cytometry; and
3. Increased lymphocyte counts with evidence of a T-cell clone.
4. Abnormalities were also found on CD4+ T cells and SS patients usually had a CD3+/CD4+/CD2−/CD7−/low T-cell activation-related immunophenotype.

In the blood by Southern blot or PCR techniques; (iii) increased lymphocyte counts with evidence of a T-cell clone and/or an aberrant loss of expression of pan-T cell markers by flow cytometry; and (iv) a chromosomally abnormal T-cell clone.

Although this algorithm is in fact an improvement over previous proposals, it does not clearly define the immunologic criteria to identify Sézary cells. Thus, our first priority was to analyze the type and incidence of the phenotypic abnormalities of Sézary cells and to define a sensitive and specific strategy to identify and quantify Sézary cells. In line with this, the International Society for Cutaneous Lymphomas has recently defined more sensitive and specific criteria to identify Sézary cells. In line with this, the International Society for Cutaneous Lymphomas has recently proposed a new algorithm for diagnosis of blood involvement in SS:

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3. Increased lymphocyte counts with evidence of a T-cell clone.
4. Abnormalities were also found on CD4+ T cells and SS patients usually had a CD3+/CD4+/CD2−/CD7−/low T-cell activation-related immunophenotype.

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Our results show that defective CD3/TCRβ, CD4, CD2 and CD7 expression are the most common phenotypic abnormalities observed on CD4+ Sézary T-cells that circulate in blood. Similar phenotypic abnormalities were also found on CD4+ T cells extracted from skin (n=3), lymph nodes (n=1) and parotid mass biopsies (n=1) (data not shown). Although the series of patients studied here did not include rare phenotypes that have been observed in SS, our results also clearly show that CD3/TCRβ−low, CD4−low, CD2−low, CD7−low is by far the most common phenotype of SS T-cells and that it clearly differs from that observed on CD4+ T cells.

**Table 3. Blood cell counts and major phenotypic data (number of total CD4+ T-cells, the CD4/CD8 ratio and the TCR-Vβ expansions found among CD4+ T-cells in the blood) in patients with reactive erythroderma (n=10).**

<table>
<thead>
<tr>
<th>Patient Number</th>
<th>Diagnosis</th>
<th>WBC (×10³/L)</th>
<th>Lymphocytes (% WBC)</th>
<th>Hemoglobin (g/dL)</th>
<th>Platelets (×10⁹/L)</th>
<th>CD4+ T-cells (% of lymphocytes)</th>
<th>CD4/CD8 ratios</th>
<th>Expanded TCR-Vβ family (% of CD4+ T-cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Atopic dermatitis</td>
<td>11.1</td>
<td>6.6</td>
<td>10.4</td>
<td>293</td>
<td>55.8</td>
<td>3.72</td>
<td>Vβ8.1+8.2 (9.9%); Vβ14.1 (56%)</td>
</tr>
<tr>
<td>2</td>
<td>Unknown</td>
<td>16.8</td>
<td>14.1</td>
<td>14.1</td>
<td>404</td>
<td>25.0</td>
<td>0.68</td>
<td>Vβ3n (21.9%); Vβ12.6 (36%)</td>
</tr>
<tr>
<td>3</td>
<td>Photodermatitis</td>
<td>19.4</td>
<td>16.9</td>
<td>11.1</td>
<td>355</td>
<td>63.8</td>
<td>2.20</td>
<td>N</td>
</tr>
<tr>
<td>4</td>
<td>Photodermatitis</td>
<td>11.8</td>
<td>15.3</td>
<td>14.0</td>
<td>174</td>
<td>62.1</td>
<td>6.36</td>
<td>Vβ5.3 (3.7%)</td>
</tr>
<tr>
<td>5</td>
<td>Photodermatitis</td>
<td>7.2</td>
<td>27.1</td>
<td>14.0</td>
<td>199</td>
<td>63.2</td>
<td>3.10</td>
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<tr>
<td>6</td>
<td>Unknown</td>
<td>12.0</td>
<td>19.8</td>
<td>12.9</td>
<td>253</td>
<td>62.9</td>
<td>4.51</td>
<td>Vβ12.1 (14.2%); Vβ12.2 (5.6%)</td>
</tr>
<tr>
<td>7</td>
<td>Chronic eosinophilic leukemia</td>
<td>4.6</td>
<td>58.2</td>
<td>11.1</td>
<td>588</td>
<td>52.0</td>
<td>1.50</td>
<td>N</td>
</tr>
<tr>
<td>8</td>
<td>Psoriasis</td>
<td>9.0</td>
<td>26.6</td>
<td>11.4</td>
<td>245</td>
<td>38.5</td>
<td>1.91</td>
<td>N</td>
</tr>
<tr>
<td>9</td>
<td>Psoriasis</td>
<td>10.3</td>
<td>17.9</td>
<td>13.1</td>
<td>401</td>
<td>35.9</td>
<td>1.84</td>
<td>Vβ8.1+8.2 (8.1%)</td>
</tr>
<tr>
<td>10</td>
<td>Toxicoderma</td>
<td>4.1</td>
<td>24.5</td>
<td>11.6</td>
<td>120</td>
<td>47.4</td>
<td>2.43</td>
<td>N</td>
</tr>
<tr>
<td>Mean ± 1 SD</td>
<td></td>
<td>9.7±3.8</td>
<td>22.7±13.9</td>
<td>12.1±1.4</td>
<td>303±138</td>
<td>50.7±13.6</td>
<td>2.83±1.66</td>
<td>9.6±8.5</td>
</tr>
</tbody>
</table>

TOR, T-cell receptor; N, Normal TCR-Vβ T-cell repertoire (no evidence for the existence of TCR-Vβ expansions); Vβni, the expanded TCR-Vβ family was not positively identified with the panel of MAb used in this study, SD, standard deviation.
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from patients with reactive erythroderma.

Despite being found in a high percentage of SS patients, none of these abnormalities is specific or sensitive enough to be used as the sole diagnostic criterion to establish a differential diagnosis between SS and reactive erythroderma. As an example, in accordance with the results presented here and in line with previous studies, decreased CD7 expression, although a common phenotypic characteristic of Sézary cells, also defines a subset of normal CD4+/CD45RO+ memory cells which can be increased in patients with benign or malignant skin diseases. Moreover, decreased CD3/TCR expression, although not observed in patients with reactive dermatitis studied in this series, has been described in conditions associated with T-cell stimulation. The same applies to other phenotypic features that had been previously found in SS, which have also been described in blood T-lymphocytes from patients with inflammatory skin diseases, including expression of cutaneous lymphocyte-associated antigen. Therefore, combined assessment of several aberrant phenotypic markers is needed. Simultaneous staining for CD7 and CD2 proved to be particularly interesting since decreased CD2 expression on CD4+/CD7+/low Sézary cells allows their discrimination from normal reactive CD4+/CD7+/low memory cells, which strongly express the CD2 molecule. In addition, decreased expression of CD3/TCRβ and CD4, although very frequent in SS, might be of limited value in cases showing low numbers of residual CD4+ T-cells once each marker is separately tested. Simultaneous staining for CD3, TCRβ CD4 and CD8 usually makes it easier to distinguish between normal CD4+/high/CD3+/high/TCRαβ+/high T-cells and CD4+/low/CD3+/low/TCRαβ+/low lymphoma cells. Furthermore, useful information obtained with this 4-color combination is provided by the fact that CD3/TCR expression on CD4+ Sézary cells is frequently decreased towards levels below those of the CD8+/TCRαβ+ T-cells present in the same sample.

In line with previous studies, CD4+ Sézary cells from the vast majority of patients, like normal CD4+ memory cells, express CD45RO+/CD45RA-, although co-expression of CD45RA was also found in a few cases. CD25 expression, although not relevant for distinguishing Sézary lymphoma cells from normal residual CD4+ T-cells, would probably be of interest in selecting patients for anti-CD25 therapy, since reactivity for the IL2 receptor α-chain on Sézary cells was found in only around half of the cases. In turn, reactivity on Sézary cells for the CD28 T-cell co-stimulatory molecule in the absence of expression of NKa molecules and killer receptors distinguishes Sézary T-cells from normal residual CTL present at relatively high levels in the same sample in some patients. Such an increased expression of NKa markers and activation-related antigens on normal residual CD8+, and to a lesser extent, CD4+ T-cells, has already been reported in the blood and in the skin of patients with CTCL and may reflect a systemic anti-tumor response. In addition, these phenotypic features might be useful in the differential diagnosis between SS and other types of T-cell lymphoma and leukemia. In
fact, although the immunophenotype that typically characterizes T-cell lymphocytic/prolymphocytic leukemia—which can also present with erythroderma—and CD4+ LGL have been previously described in detail and clearly differ from that described for SS, defective CD3 and CD7 expression may also be found in peripheral T-cell lymphomas other than SS. The same applies to other phenotypic features that have been previously found in SS, such as absence of CD26 expression, a marker that is also frequently negative in other peripheral T-cell lymphomas. Thus, additional studies should be carried out in order to determine whether the unique immunophenotypic features described here are specific to Sézary cells and allow differential diagnosis between SS and other T-cell neoplasias.

Despite the similarities between patients with SS, it should be noted that important intra-tumor phenotypic heterogeneity was frequently observed, this mainly involving the presence of large and small neoplastic cells and subsets of Sézary cells expressing different levels of CD7 and/or CD2.

Combined immunophenotypic and DNA studies proved to be of a particular interest in that they showed that the presence of large CD4+ Sézary cells is predictive for a hyperdiploid—typically nearly tetraploid—DNA content, whereas small Sézary cells usually have a diploid, or less frequently hypodiploid, DNA content. Although previous reports have already shown that large CD4+ Sézary cells may display an aneuploid DNA content, to the best of our knowledge this is the first report in which the overall frequency at which these cells appear in the blood of newly diagnosed patients with SS, as well as their distribution within the small and large neoplastic CD4+ T cells subsets, are reported.

Despite co-existence of DNA diploid and hyperdiploid CD4+ T-cell populations in a relatively high proportion of cases, associated or not with phenotypic heterogeneity, molecular studies were consistent with a (mono)clonal T-cell proliferation. The fact that the proportion of cases with large Sézary cells was higher in patients with a long (> 1 year) past history of symptoms, together with the phenotypic changes observed in some cases during follow-up, would support the notion that intra-tumor heterogeneity is a consequence of ongoing somatic mutations and acquisition of new chromosomal abnormalities developing over time. The occurrence of large cell transformation in patients with CTCL is well documented. In one such case large Sézary cells proved to be clonally identical to the original CTCL and in another case large Sézary cells were shown to be hyperdiploid. Intra-tumor diversity may influence response to therapy, as indeed occurred in one of the cases included in this series (patient #10), whose large Sézary cells preferentially responded to treatment with 2-deoxycoformycin.

Another goal of the present study was to explore the utility of immunophenotypic analysis of the TCR-β repertoire, either in confirming the (mono)clonality of a phenotypically abnormal T-cell population or in facilitating direct identification of residual tumor cells, even when they are present in blood at very low frequencies. Our results confirm that TCR-β repertoire studies are of great utility for detecting (mono)clonality of aberrant CD4+ T cells in SS, once previously established criteria have been applied. Nevertheless, the fact that the expanded TCR-β family was not directly identified in most cases limits the use of a combined strategy—simultaneous assessment of aberrant markers with a specific TCR-β family—for identifying residual tumor cells until the panel of anti-TCR-β antibodies available is completed. The fact that the clonally expanded TCR-β family was not identified in 78% of cases using a panel of 24 anti-TCR-β MAb that identify around 60% of all normal blood CD4+ T-cells would suggest that Sézary cells preferentially bear one or more of the few TCR-β families that were not represented in the panel of MAB used in this study. This notion is also supported by the results obtained here in patients with reactive dermatitis and those previously described with acute infectious mononucleosis with the same panel of MAb, in which the expanded TCR-β families found corresponded to those represented most in normal individuals. The hypothesis of a preferential TCR-β usage by Sézary cells is further supported by the fact that in 3 out of the 4 cases in which the expanded TCR-β family was identified (corresponding to 17% of all cases studied), the same TCR-β family (TCR-β 13.6) was found, this particular TCR-β family being typically expressed in < 2% of normal blood CD4+ T cells. Studies published to date aimed at the characterization of the TCR-β repertoire in CTCL are scarce, most of them including CTCL other than SS and are mainly based on molecular studies using primers specific for the TCR-β families, and performed in skin biopsies. From those studies in which anti-TCR-β MAb were used to identify clonal cutaneous T-cell infiltrates by immunohistochemical methods, it could be concluded that the TCR-β-restricted T-cell clones corresponded to the phenotypically abnormal T-cell populations and a wide range of TCR-β families were found to be expanded in line with the results from molecular studies. Despite this, Gorochov et al. showed restricted TCR-β repertoire usage of five TCR-β families (Vβ5, Vβ6, Vβ8, Vβ13, and Vβ18) in the blood of 16 SS patients using both anti-TCR-β MAb and PCR-techniques. Such discrepancies
probably relate to the criteria used to enter patients (SS versus other CTCL) and samples (skin versus blood) into the different studies, to the use of different techniques (molecular versus immunologic) to analyze the TCR-β repertoire and to different technical approaches for the same technique (analysis of the TCR-β repertoire on total CD3+ T cells versus differential analysis on CD4+ and CD8- T-cell subsets). Another important factor that should be taken into account when comparing the results of these studies concerns the occurrence of TCR-β expansions among normal blood CD8+ and CD4+ T cells, as they may be erroneously taken as corresponding to neoplastic cells, particularly in cases in which the fraction of CTL predominates over that of circulating Sézary cells or when highly sensitive methods, such as PCR-based techniques, are used. In line with this, it should also be noted that a restricted TCR-β repertoire has been shown to occur in the blood of reactive skin conditions that may mimic CTCL, including atopic dermatitis, and a TCR-β restricted CTL response has also been described in the skin of patients with CTCL. Thus, as shown in the present report, assessment of the TCR-β repertoire specifically on the phenotypically aberrant CD4+ T cells may be of a great help in identifying TCR-β-restricted lymphoma cells and in distinguishing them from other polyclonal, oligoclonal or monoclonal T-cell lymphoma cells and in distinguishing them from other polyclonal, oligoclonal or monoclonal T-cell lymphoma cells and in distinguishing them from other polyclonal, oligoclonal or monoclonal T-cell lymphoma cells.

In summary, our results indicate that the presence of a TCR-β restricted CD28-/CD5-/NKa-CD4+ T-cell population expressing abnormally low levels of CD3, TCRβ, CD4, CD7 and/or CD2 molecules would support the diagnosis of SS in patients with erythroderma. Further studies in larger series of patients are required in order to cover all the abnormal immunophenotypic patterns that may be associated with SS and to establish the value of these criteria for the differential diagnosis between SS and other T-cell lymphomas/leukemias. In addition, assessment of the TCR-β families not analyzed in this study is necessary to fully identify those specific TCR-β families preferentially used by Sézary cells.

References

Blood involvement in Sézary syndrome

Pre-publication Report & Outcomes of Peer Review

Contributions
ML, JA; conception and design, analysis and interpretation of data, drafting the article and revising it critically for important intellectual content; final approval of the version to be published; MdAT: conception and design; MLQ: conception and design; AHS: conception and design; SF: conception and design and analysis and interpretation of data, drafting the article and revising it critically for important intellectual content; final approval of the version to be published.

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In the following paragraphs, Dr. Matutes summarizes the peer-review process and its outcomes.

What is already known on this topic
It is well recognised that Sézary cells express aberrant phenotypes and are heterogeneous regarding ploidy shown by cytogenetics and DNA analysis.

What this study adds
The present study confirms that blood circulating Sézary cells have aberrant phenotypes allowing them to be distinguished from lymphocytes of patients with reactive dermatitis and that they have different ploidy in relation to the cell size. In addition, and within some patients, there is phenotypic heterogeneity. By using a large set of monoclonal antibodies against the TCR beta families, the authors are able to demonstrate clonality in all patients and suggest that this approach may avoid the need of molecular methods. Nevertheless this needs to be validated in a higher number of cases.