

The Usefulness of CD26 in Flow Cytometric Analysis of Peripheral Blood in Sézary Syndrome

Katalin Kelemen, MD, PhD,¹ Joan Guitart, MD,² Timothy M. Kuzel, MD,³ Charles L. Goolsby, MD,¹ and LoAnn C. Peterson, MD¹

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Abstract

The loss of CD26 expression was proposed to be a constant feature of circulating Sézary cells by flow cytometric immunophenotyping (FCIP), but the experience with CD26 is limited. To establish its usefulness, CD26 results were correlated with morphologic, molecular, and immunophenotypic findings. Based on FCIP of 179 samples of peripheral blood, CD26 negativity was found in 59.3% of cases with Sézary syndrome (SS), 33.3% of mycosis fungoides (MF), 14.2% of benign dermatosis (BD), and no control cases. In diagnostic subgroups of SS based on morphologic, molecular, and immunophenotypic criteria, the percentage of CD26– cases varied from 41.1% to 63.6%. The specificity of a CD26– result was inferior to that of T-cell antigen loss in differentiating SS from MF and BD. CD26 offers lower diagnostic performance than previously suggested; however, in addition to the findings of major T-cell antigen loss, it could improve sensitivity of FCIP in patients with SS.

Sézary syndrome (SS) is the leukemic variant of cutaneous T-cell lymphoma, characterized by the triad of erythema, generalized lymphadenopathy, and the presence of neoplastic T cells (Sézary cells) in skin, lymph nodes, and peripheral blood. Circulating Sézary cells were originally identified by Sézary and Bouvrain¹ in 1938 as atypical mononuclear cells (“cellules monstreuses”) in a patient with chronic erythroderma and skin histologic findings suggestive of mycosis fungoides (MF). Approximately 30 years later, the distinctive grooved nucleus was described and became the morphologic hallmark of the Sézary cell.^{2,3}

The degree of peripheral blood involvement by cutaneous T-cell lymphoma cells is an important prognostic variable because SS is generally regarded to have a worse prognosis than “aleukemic” erythrodermic cutaneous T-cell lymphomas not involving the peripheral blood.^{4,5} However, the recognition of SS has been difficult because there is no universally accepted method to identify and quantify the neoplastic cells. The morphologic identification of Sézary cells in the peripheral blood requires special expertise, and even the most experienced observer may significantly underestimate the number of neoplastic cells.⁶ Furthermore, lymphocytes with convoluted, cerebriform nuclei may be seen in advanced MF, various reactive skin conditions, and even in normal peripheral blood.^{7–11}

Flow cytometric immunophenotyping (FCIP) has become a useful tool in the detection of circulating Sézary cells, but a highly sensitive and specific marker of the neoplastic Sézary cells is yet to be identified. The most commonly reported phenotypic aberrancy in SS is the loss of CD7, whereas diminished or absent expression of CD2 or CD3 is less frequently observed.^{12–14} Unfortunately, some of these

phenotypic changes, especially down-regulation of CD7 expression, may be commonly seen in reactive T-cell populations.¹⁵ Furthermore, the cell population with loss of CD7 expression might not overlap with the neoplastic cells because circulating clonal cells from patients with SS identified by T-cell receptor (TCR) polymerase chain reaction (PCR) have been reported in CD4+/CD7- and CD4+/CD7+ subpopulations of T cells.¹⁶

The lack of CD26 (dipeptidyl-aminopeptidase IV) was proposed to be a constant feature of circulating Sézary cells.^{4,17} CD26 is a glycosylated membrane protease that cleaves Xaa-pro or Xaa-Ala dipeptides from the N-terminus of polypeptides. It is constitutively expressed on endothelial and epithelial cells of various tissues and on more than 50% of peripheral blood lymphocytes in healthy subjects.¹⁸ Previous studies suggested that a CD4+/CD26- percentage higher than 30% of peripheral blood lymphocytes could correctly identify the presence of Sézary cells in the blood of patients with SS or MF. The experience with the CD26 marker remains limited, and diagnostic usefulness remains to be established.

The goal of our study was to evaluate the usefulness of CD26 in flow cytometric evaluation of peripheral blood samples from patients with SS by correlating CD26 expression with morphologic and immunophenotypic criteria, following the most recently recommended criteria of the International Society for Cutaneous Lymphoma (ISCL). In addition, we analyzed the individual and combined diagnostic usefulness of major T-cell antigen loss and the loss of CD26 expression in flow cytometric analysis of peripheral blood samples in SS.

Materials and Methods

The study was approved by the institutional review board of Northwestern Memorial Hospital (NMH), Chicago, IL. All studies were done retrospectively based on review of the medical records of NMH and Northwestern Medical Faculty Foundation.

Cases

FCIP analyses were performed on 134 peripheral blood samples from 44 patients with clinical indications of SS or "rule out Sézary Syndrome" at NMH between January 2004 and November 2006. In addition, peripheral blood samples of 45 adult control subjects with normal CBC and differential counts detected on the same day were also analyzed by FCIP using the same panel of antibodies. The results of the morphologic examinations of the peripheral blood for Sézary cells and, when performed on the peripheral blood within 24 hours of the FCIP analysis, the results of TCR gene rearrangements by PCR were also reviewed.

Sézary Cell Counts

Sézary cell counts were performed by senior technologists in the hematology laboratory. Peripheral blood smears were stained with Wright-Giemsa stains, and "Sézary cells" were counted by identifying lymphocytes with folded and grooved nuclear membranes (cerebriform nuclei). At least 600 lymphocytes were routinely counted on 3 smears in each case (200 per smear). The results were reviewed and confirmed by a staff hematopathologist, and the results were reported as the absolute number of Sézary cells per microliter of blood and as the percentage of peripheral lymphocytes.

Flow Cytometric Immunophenotyping

FCIP was performed as described previously.¹⁹ Briefly, peripheral blood was collected in EDTA or sodium heparin as anticoagulant. T-cell immunophenotyping was performed within 24 hours of collection by using the following fluorochrome-conjugated antibodies: CD2-allophycocyanin (APC), CD4-Cy5.5, CD5-peridinin chlorophyll protein-Cy5.5, CD25-APC-Cy7 (M-A251; Becton Dickinson Biosciences, San Jose, CA); and CD3-PC7, CD8-APC, and CD26-phycoerythrin (Beckman Coulter Immunotech, Marseille, France). Following RBC lysis and washing in phosphate-buffered saline, the peripheral blood leukocytes were incubated with antibody at room temperature in phosphate-buffered saline containing 30% newborn calf serum (Gibco, Invitrogen, Carlsbad, CA). All antibodies were titrated to yield the highest signal (positive T-cell staining)-to-noise (background staining level) separation.

Data collection and analysis were performed on a Becton Dickinson LSRII flow cytometer using 488- and 633-nm lasers. For the analysis of major T cell-associated antigens and CD26, an arbitrary cursor was established based on a comparison with background staining observed on the negative granulocyte, monocyte, and B-cell populations, which all showed virtually the same level of background staining under the given staining conditions. Although staining patterns heavily overlapping with the negative region so defined were considered dim positive, for the purposes of this study and comparison with other published work, the fraction of the T-cell population that showed overlapping staining characteristics with the background was considered negative.

TCR Gene Rearrangement by PCR

PCR analysis was performed by using a TCRG Gene Clonality Assay kit (InVivoScribe Technologies, San Diego, CA).²⁰ Briefly, 0.5 to 1 µg of genomic DNA was amplified in duplicate with 0.15 µL of *Taq* Polymerase-Gold (ABI, Foster City, CA) and a mixture of fluorochrome-labeled primers specific for conserved regions within the variable (V) regions V_γ1 through V_γ8, V_γ10, V_γ9, and V_γ11 and the J_γ exon of the joining (J) region of the TCR gene. A TCR clonal control was used

for the positive control sample and normal peripheral blood as the negative control sample. We performed 35 cycles on a thermocycler, and the PCR products were analyzed on an ABI 310 device (ABI). For the *TCR* PCR result to be considered a clonal rearrangement, the highest peak had to be at least 3 times higher than the third highest peak in the set of PCR products. Peaks that were 2 to 3 times higher were called indeterminate.

Statistical Analyses

Statistical analyses were performed using the Wilcoxon rank sum test and the Bonferroni correction for multiple testing.

Results

The clinical and laboratory features of the various study groups are summarized in **Table 1**. We studied 134 peripheral blood samples from 44 patients submitted for FCIP with a clinical indication of SS or rule out Sézary syndrome. In 28 patients, the diagnosis of SS was made based on the presence of one or more of the following criteria in the appropriate clinical context: an absolute Sézary cell count greater than or equal to 1,000/ μ L of blood, a percentage of Sézary cells greater than or equal to 20% of the total lymphocyte count, a CD4/CD8 ratio greater than 10 by FCIP, and the demonstration of clonal *TCR* gene rearrangement by PCR. Among the 28 patients with SS, samples from 5 were first studied at diagnosis; for the remaining 23 patients, the diagnosis of SS had been established previously. All of these 23 patients had received therapy, including psoralen plus UV-A combined with interferon or bexarotene in 18, alemtuzumab in 9, lenalidomide in 4, denileukin diftitox in 4, combination chemotherapy in 3, and allogeneic stem cell transplantation in 2. The remaining 16 patients did not satisfy criteria for SS at the time of FCIP. This group included 9 patients with MF and 7 with benign dermatoses, such as lymphomatoid papulosis (3

cases), systemic lupus erythematosus (SLE; 1 case), parapsoriasis (1 case), and generalized pruritus (2 cases).

Sézary Cell Counting

Convoluting lymphocytes were identified in all study groups, including 12 control subjects (Table 1). In the control group, the percentage of convoluting lymphocytes ranged from 1.0% to 6.0% (mean, 3.3%), and the absolute Sézary cell counts ranged from 15 to 124/ μ L (mean, 74.2/ μ L).

In cases with SS, the percentage of Sézary cells and the absolute number of Sézary cells was significantly higher than in the control group, when compared by using the Wilcoxon rank sum test ($P < .0001$). The proposed ISCL criteria for peripheral blood involvement by SS include an absolute Sézary cell count of greater than 1,000/ μ L.²¹ Other groups have advocated Sézary cell counts greater than or equal to 20% of the total number of lymphocytes as a criterion for the diagnosis.^{4,16} These morphologic criteria for SS were met by 33 (29.5%) and 68 (60.7%) of the SS cases, respectively. In contrast, none of the cases from the control group satisfied either criterion.

TCR Gene Rearrangement by PCR

The results of *TCR* gene rearrangement studies were considered for correlation with the hematologic and immunophenotypic results only when they were performed within 24 hours of FCIP. In the SS group, 24 PCR tests (representing 13 patients) were performed on peripheral blood samples for *TCR* gene rearrangement within 24 hours of FCIP. Of the 24 tests, 20 yielded positive results (19 clonal and 1 oligoclonal), 3 were negative, and 1 was indeterminate. In the case with the oligoclonal result, one of the peaks appeared identical to a peak previously demonstrated by *TCR* PCR in a skin sample and a peripheral blood sample from the same patient. In the MF patient group, 1 of 5 *TCR* gene rearrangement studies was positive in the peripheral blood. Two of the patients with benign dermatosis also had clonal *TCR* rearrangement in the peripheral blood (Table 1).

Table 1
Demographic and Laboratory Characteristics of Study Groups

	Sézary Syndrome (n = 28)	Mycosis Fungoides (n = 9)	Benign Dermatoses (n = 7)	Control Group (n = 45)
No. of flow cytometric analyses	112	14	8	45
Mean age (range), y	61 (41-82)	59 (22-85)	56 (35-73)	Adult
M/F ratio	15/13	5/4	5/2	Not studied
Mean WBC count, / μ L ($\times 10^9$ /L)	11,900 (11.9)	6,420 (6.42)	6,900 (6.9)	<8,000 (8.0)
Mean Sézary cells (%)	35.8	4.3	2.5	3.3*
Mean No. of Sézary cells (range), / μ L	2,641.0 (0-120,000)	59.5 (0-334)	72.6 (0-243)	74.2 (0-124)*
Mean CD4/CD8 ratio	21.5	2.4	1.3	Not studied
Clonal <i>TCR</i> , No. positive/No. tested	20/24	1/5	2/2	Not studied

TCR, T-cell receptor.

* n = 12.

Flow Cytometric Immunophenotyping

CD4/CD8 Ratio

An increase in circulating CD4+ T cells resulting in a CD4/CD8 ratio of greater than 10 has been proposed by ISCL as another criterion for the diagnosis of SS.²¹ The mean and median CD4/CD8 ratios were significantly higher in the SS group compared with other groups (Table 1). A CD4/CD8 ratio of greater than 10, however, was only observed in 39% of the analyses (38/97) in SS samples. In contrast, the highest observed CD4/CD8 ratio observed in all other groups was 5.6.

Major T-Cell Antigen Aberrancies

Antigen expression by peripheral T cells was analyzed by 4-color FCIP in all study groups, including 45 adult control cases. In the control cases, the CD7 antigen showed heterogeneous staining from dim to bright. Although this was considered dim positive and an accurate determination of the percentage of negative and positive is not possible, for the purposes of this study and comparison with other published results, an arbitrary cursor for the determination of the percentage of events in the two regions was defined as described in the "Materials and Methods" section. In the control samples, the fraction of the T-cell population that showed overlapping CD7 staining characteristics with the background (CD7-) varied between 2.7% and 28.0% of the T cells (mean, 10.63%; median, 9.8%; SD, 6.3%).

Of the 134 samples submitted with a clinical indication of SS or rule out SS, 112 represented patients with SS. In this group, no aberrancy was observed in 11 (9.8%) of 112 analyses. The most commonly observed aberrancy was a partial or complete loss of CD7 as the only aberrancy, observed in 86 (76.8%) of 112 analyses. Other much less frequently observed anomalies included a partial loss or diminished intensity of CD2, a combination of diminished staining of CD7 and CD2, diminished CD7 staining with distinctly dimmer CD2 intensity, diminished CD7 staining with dimmer CD3, and diminished CD7 staining with dimmer CD2 and CD3 intensity (Table 2).

Table 2
Aberrancies of Major T-Cell Antigen (CD2, CD3, CD5, and CD7) Expression in 112 Flow Cytometric Analyses of Peripheral Blood Samples in Patients With Sézary Syndrome

	No. (%) With Feature
No T-cell antigen deletion	11 (9.8)
CD7 deletion only	86 (76.8)
CD2 deletion only	7 (6.3)
CD2 and CD7 deletion	3 (2.7)
CD7 deletion and discretely dimmer CD2	2 (1.8)
CD7 deletion and discretely dimmer CD3	2 (1.8)
CD7 deletion and dimmer CD2 and CD3	1 (0.9)

Extent of Major T-Cell Antigen Loss and Correlation With the Diagnosis of SS

The extent of the major T-cell antigen loss was quantified and expressed as percentage of T cells. The median of major T-cell antigen loss was significantly higher in the SS group than in any other group when using the Wilcoxon rank sum test (Figure 1A). By using the same statistical analysis, the median of the MF group was not significantly different from that of the control group ($P < .49$); however, the median of the benign dermatosis group was significantly different ($P < .047$). There were no significant differences among the MF, benign dermatosis, and control groups when using the Bonferroni correction for multiple testing.

The correlation between the extent of T-cell antigen loss and the diagnosis of SS was analyzed further by establishing the percentage of cases qualifying for SS (based on the diagnostic criteria as listed in the "Materials and Methods" section) in different ranges of T-cell antigen loss (Figure 1B). The percentage of cases diagnosed as SS increased gradually with increasing extent of T-cell antigen loss, with a considerable increase in the percentage of SS cases with more than 50% of T cells showing T-cell antigen loss. These data show that the increasing extent of major T-cell antigen loss correlates with increasing identification of SS cases and suggest that T cells with down-regulated T-cell antigens are representative of neoplastic Sézary cells. Of note, none of the control, MF, or benign dermatosis cases had a T-cell antigen loss greater than 30%. A threshold of 30% of T-cell antigen loss would represent a sensitivity of 72%, with 100% specificity with regard to MF, benign dermatosis, and control cases. Raising the threshold to 40% would result in a loss of sensitivity to identify SS (59%).

CD26 Expression

In control cases, CD26 exhibited heterogeneous staining from negative to positive ("smear") and was expressed on 44% of T lymphocytes (range, 14%-84%; median, 44%; mean, 44%). Expression was completely confined to the CD3+ T-cell population. In this study, the loss of CD26 expression (CD26-) was defined as a well-formed cluster of T lymphocytes with the entire analyzed population showing CD26 staining overlapping with the background. The percentage of CD26- results was higher in the SS cases than in MF, benign dermatosis, or control cases (Figure 2A), with a sensitivity to identify SS of 59% and a specificity of 66%, 86%, and 100% with regard to MF, benign dermatosis, and control cases, respectively.

To ascertain the diagnostic relevance of the loss of CD26 expression in SS, we analyzed the percentage of CD26- results in different diagnostic subgroups of SS as defined by the criteria listed in the "Materials and Methods" section and with the extent of T-cell antigen aberrancies. Sézary cell counting and TCR gene rearrangement studies by PCR had to

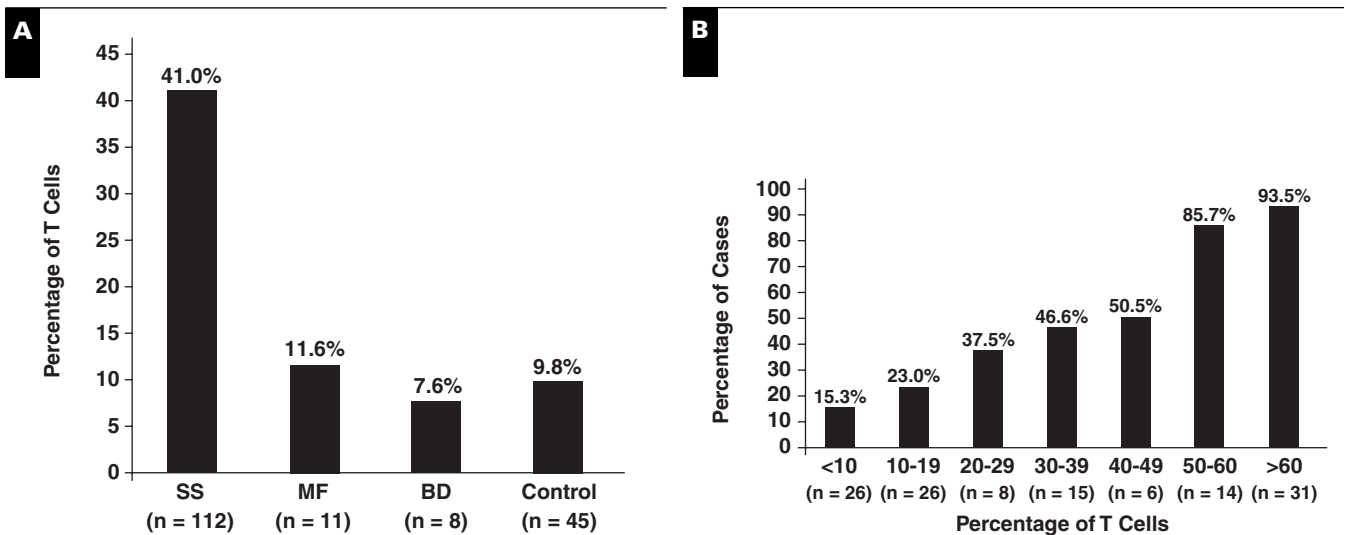


Figure 1 **A**, The extent of major T-cell antigen (CD2, CD3, CD5, and CD7) loss, expressed as percentage of T cells, is compared among Sézary syndrome (SS), mycosis fungoides (MF), benign dermatosis (BD), and control cases. The number of flow cytometric analyses in each group is shown. In the SS cases, the median of T-cell antigen loss was significantly higher than in the other 3 groups ($P = .0001$ vs MF group; $P < .0001$ vs BD and control groups). The MF, BD, and control groups were not significantly different. **B**, The percentage of cases that qualify for SS in different ranges of T-cell antigen loss. The extent of T-cell antigen loss is expressed as percentage of T cells. The number of cases (n) falling into each range of the extent of T-cell antigen loss is shown. The probability of the diagnosis of SS increases with increasing extent of T-cell antigen loss, suggesting that the T cells with loss of major T-cell antigens are representative of the neoplastic cells in SS.

be performed within 24 hours of the FCIP to be considered for correlation. In all diagnostic subgroups of SS, the percentage of CD26⁺ cases remained relatively stable at about 60% (59.3%-63.6%) except in the group in which SS was defined by TCR gene rearrangement, for which the percentage of CD26⁺ cases was considerably lower (41.1%) (Figure 2B). In summary, in all diagnostic subgroups of SS, at least 40% of the cases retained CD26⁺ staining.

Correlation of the loss of CD26 expression with the extent of T-cell antigen aberrancies is shown in Figure 2C. When T-cell antigen anomalies extended to only 0% to 30% of T cells, CD26⁺ results were observed in no more than 30.7% of cases. A considerable increase was observed in the percentage of CD26⁺ cases when the T-cell antigen loss extended beyond 30% of T cells. Further extension of the T-cell antigen loss to more than 40%, 50%, or 60% of T cells, however, was not accompanied by increase in the percentage of CD26⁺ results; instead the percentage remained relatively stable in the range of 57.1% to 66.6%. We analyzed the distribution of the CD26⁺ T cells in terms of their overlap with major T-cell antigen loss. In 55% of CD26⁺ cases, the loss of CD26 expression was observed in all T cells. In the remaining 45%, however, the loss of CD26 expression was exclusively in the T cells that also exhibited loss of CD7 expression; the CD7⁺ T cells retained their dim CD26⁺ staining. No cases showed loss of CD26 restricted to the CD7⁺ T-cell population.

Figure 3 shows the individual and additive diagnostic performance (sensitivity and specificity) of the major T-cell antigen aberrancies and the loss of CD26 expression in the diagnosis of SS in comparison with the other study groups, MF, benign dermatosis, and control cases. For major T-cell antigen loss, a threshold of 30% is used, and only cases that qualify for SS based on one or more of the diagnostic criteria are considered. The sensitivity for T-cell antigen loss of more than 30% to identify SS is 72% (specificity is 100% with regard to MF, benign dermatosis, and control cases). The sensitivity of CD26 negativity to identify SS is 61% (specificity, 79%, 88%, and 100% with regard to MF, benign dermatosis, and control cases, respectively). A higher sensitivity of 85% can be achieved in the diagnosis of SS by combining the 2 markers, with some loss of specificity with regard to MF (72%) and benign dermatosis (88%) and with 100% specificity with regard to control cases.

Discussion

Criteria for the diagnosis of SS should reflect an increased blood tumor burden because the degree of peripheral blood involvement is an important prognostic factor.²¹ In a recent report of the ISCL, criteria recommended for the diagnosis of SS include one or more of the following: an absolute

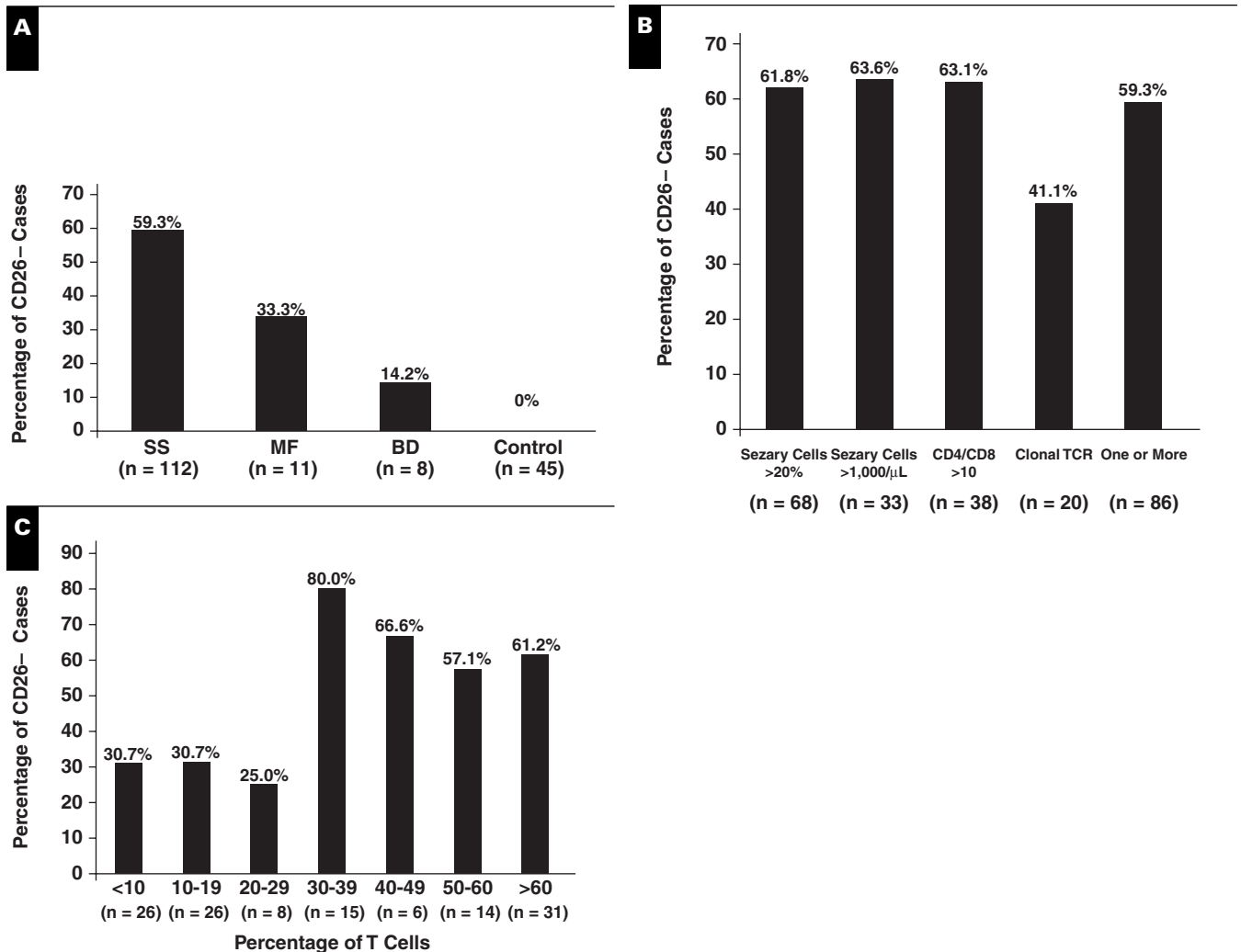


Figure 2 **A**, The percentage of CD26- cases in Sézary syndrome (SS), mycosis fungoides (MF), benign dermatosis (BD), and control cases. The number of flow cytometric analyses (n) in each group is shown. The percentage of CD26- results is higher in the SS group than in the other groups (sensitivity for SS, 59%); however, the loss of CD26 is not entirely specific for SS because 33% and 14% of cases were CD26- in the MF and BD groups, respectively. No CD26- result was detected in control cases. **B**, The percentage of CD26- cases in distinct diagnostic subgroups of SS, defined by the following criteria: percentage of Sézary cells >20%, absolute number of Sézary cells >1,000/ μ L of blood, CD4/CD8 ratio greater than 10, the presence of clonal T-cell receptor (TCR) gene rearrangement detected in peripheral blood by polymerase chain reaction, or one or more of the aforementioned criteria. The percentage of CD26- results is very similar in the subgroups (62%-64%) except in the subgroup defined by molecular criteria, in which it is considerably lower (41%). **C**, The correlation of CD26 negativity with the extent of major T-cell antigen (CD2, CD3, CD5, and CD7) loss. The percentage of CD26- cases is shown in different ranges of extent of T-cell antigen loss. T-cell antigen loss is expressed as a percentage of T cells, and the number of flow cytometric analyses (n) is shown for each range. The percentage of CD26- cases shows a considerable increase at 30% or greater T-cell antigen loss but does not show a further increase beyond 40% of T-cell antigen loss, at which point it remains relatively stable in the range of 57% to 66%.

Sézary count of at least 1,000/ μ L, demonstration of immunophenotypic abnormalities by flow cytometry (such as an expanded CD4+ T-cell population resulting in a CD4/CD8 ratio of greater than 10, loss of any or all of the T-cell antigens [CD2, CD3, CD4, CD5], or both), and/or increased lymphocyte counts with evidence of a T-cell clone in the blood by

Southern blot, PCR, or a cytogenetic method.²¹ Although the usefulness of FCIP appears unquestionable, it is unclear that additional immunophenotypic criteria are appropriate for the diagnosis of SS, eg, the percentage of CD4+ T cells exhibiting loss of CD7 or the role of CD4+/CD26- flow cytometric results.

The aim of our study was to investigate the usefulness of CD26 in SS by flow cytometry, by correlating the loss of CD26 expression with hematologic and other immunophenotypic findings, including the loss of major T-cell antigens. We studied 179 FCIP analyses including CD26 representing 28 patients with SS (112 analyses), 9 patients with MF (14 analyses), 7 patients with benign dermatosis (8 analyses), and 45 adult control subjects (45 analyses).

Morphologic correlation was available in all cases of SS, MF, and benign dermatosis and in 12 control cases. Lymphocytes with convoluted nuclei were observed in all study groups, including the control group. The absolute Sézary cell counts, however, were significantly higher in the SS group than in the other groups, whereas the counts in the MF and BD groups were not significantly different from those in the control cases. In addition to the proposed ISCL criterion of 1,000 or more Sézary cells/ μ L of blood, we also used an alternative criterion of Sézary cells exceeding 20% of the total lymphocytes. In the past, several authors recommended the percentage of the total lymphocyte count as an alternative to absolute Sézary counts with different threshold values to be considered diagnostic of SS, such as 5%,²² 15%,²³ and 20%.^{24,25} Current experience indicates, however, that severe benign erythrodermas occasionally have Sézary cell percentages exceeding 20%.²⁶⁻²⁹ Therefore, 20% or more Sézary cells in the lymphocyte population per se might not be an adequate diagnostic criterion for SS because of the potential for false-positive results, but it remains useful for screening purposes because of its high sensitivity. In our benign dermatosis cases, we did not observe such a high percentage of convoluted lymphocytes, but our number of cases in this category was small. In our study, the 20% cutoff was found to have similar specificity to the numeric criterion and, as expected, had a higher sensitivity.

Immunophenotypic abnormalities were identified in 90% of analyses in the SS group. A CD4/CD8 ratio of more than 10 was observed only in 39% of the cases in the SS group. This frequency is considerably lower than that observed by the ISCL Workshop²¹ but similar to that in another recent study³⁰ in which the majority of patients with SS had received therapy. Because in our study 23 of 28 patients with SS were previously diagnosed and treated for SS, a decreased tumor burden might account for the discrepancy because the criterion of a CD4/CD8 ratio greater than 10 is highly dependent on tumor burden.

In terms of major T-cell antigen loss, the most commonly observed aberrancy was the loss or decrease of CD7 expression (76.8% of cases). Other rarely encountered aberrancies included loss or down-regulation of CD2 and a combination of decreased CD7 staining with dimmer CD2, dimmer CD3, or both. Because down-regulated expression of CD7 is a well-known characteristic of reactive T-cell populations,¹⁵ the loss

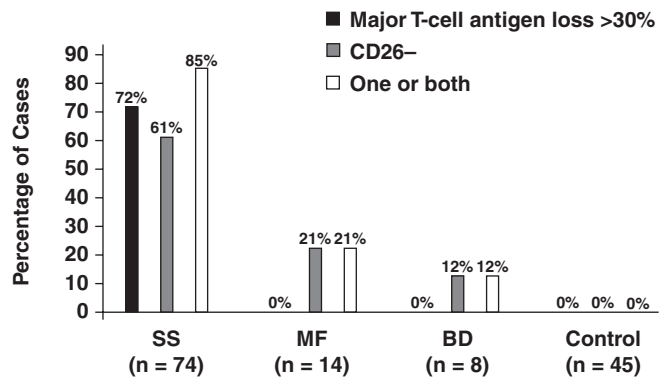


Figure 3 Individual and additive diagnostic performance of major T-cell antigen loss (threshold >30%) and CD26 negativity in Sézary syndrome (SS), mycosis fungoides (MF), benign dermatoses (BD), and control cases. The number of flow cytometric analyses (n) is shown for each group. In the SS group, only cases that qualify for SS based on one or more of the criteria are considered. Greater than 30% major T-cell antigen loss was reached in 72% of SS cases and in none of the cases in the MF, BD, and control groups (sensitivity, 72%; specificity, 100%). A CD26– result was observed in 45 SS cases (61%), 3 MF cases (21%), 1 BD case (12%), and no control cases (sensitivity, 61%; specificity, 79%, 88%, and 100% with regard to MF, BD, and control cases, respectively). A combination of the 2 approaches increased the sensitivity to identify SS to 85%.

of CD7 expression is not meaningful unless the extent of antigen loss in terms of the CD4+ T cells is considered.

Harmon et al¹⁴ and Bernengo et al⁴ found that 46% and 55% of patients with SS, respectively, had 40% or more CD4+/CD7– cells in the blood. None of the patients with benign inflammatory skin diseases reached this threshold in their studies. Moreover, Laetsch et al³¹ found a significant correlation between the percentage of CD4+/CD7– cells and Sézary cell counts. Similarly, Rappl et al³² found that the CD4+/CD7– T cells compose the dominant T-cell clone in the peripheral blood in patients with SS. Another study raised concern about the significance of the CD7– T cells by demonstrating that the expanded CD4+/CD7– population in some cases does not represent the dominant T-cell clone.¹⁶ The ISCL currently recommends a tentative blood criterion of 40% or more of CD4+/CD7– cells.

In our study, the extent of T-cell antigen loss was significantly higher in the SS group than in the MF, benign dermatosis, and control groups. In addition, the increasing extent of T-cell antigen loss correlated with an increasing percentage of SS diagnoses, suggesting that the CD4+/CD7– T cells are representative of the neoplastic cells. At the recommended threshold of 40% or more CD4+/CD7– cells, 59% of our SS

cases satisfied this value, a percentage slightly higher but close to the results detected by Bernengo et al.⁴ In our study, however, a threshold of greater than 30% appeared just as specific as 40% because none of the control, benign dermatosis, or MF cases reached a value of greater than 30% of CD4+/CD7– cells vs 72% of the SS cases that showed a value of 30% or more (sensitivity, 71.6%; specificity, 100%). These data suggest that in our laboratory, the recommended threshold of 40% CD4+/CD7– cells is highly specific but less sensitive than 30%. Rather than following preset criteria, it might be more useful for individual laboratories to establish a threshold based on their own patient populations.

Substantially fewer data are available in regard to the diagnostic performance of the CD26 marker. Bernengo et al.³³ suggested that a CD4+/CD26– percentage value higher than 30% of peripheral blood lymphocytes could correctly identify the presence of Sézary cells in the blood of patients with SS or MF. This finding was in contrast with that for the peripheral blood of healthy control subjects, in which CD26 was expressed on $57.9\% \pm 1.1\%$ of CD3+ peripheral blood lymphocytes.

In our laboratory, however, CD26 was expressed in only about 44% of CD3+ lymphocytes of adult control subjects, with 56% (range, 17%-85%) showing overlap with background staining (CD26–), and a CD4+/CD26– percentage value higher than 30% was observed in 85% of control cases. Thus, we considered CD26 as negative only when the analyzed lymphocytes formed a distinct cluster with entirely overlapping staining characteristics with the background. The differences in the baseline staining characteristics can be attributed to different instrumentation, different monoclonal antibody, or different fluorochromes used; higher blocking reagent concentrations in our staining protocols; and, most important, the fact that most of the previous studies considered an isotype-matched negative control to set the location of the cursor for CD26 staining, whereas we considered the staining of internal biologically negative populations (eg, B cells and granulocytes) as negative control populations.^{33,34}

Loss of CD26 expression was observed in 59.3% of SS cases, 33.3% of MF group, 14.2% of benign dermatosis, and none of the control cases (Figure 2A). Similar results were obtained when analyzing the percentage of CD26– cases in the diagnostic subgroups of SS defined by the distinct ISCL criteria (sensitivity varied from 59.3%-63.6%) except in the group in which SS is defined by the presence of a clonal *TCR* gene rearrangement (only 41.1%).

Because the performance of flow cytometry depends on the availability, or load, of abnormal cells for analysis, it is predictable that it correlates better with hematologic parameters than with molecular results. The criterion of the clonal *TCR* gene rearrangement by molecular methods, unlike hematologic criteria, is independent of the tumor burden and is much more sensitive and, thus, is more efficient to identify

cases with minimal residual disease. The presence of a T-cell clone in the peripheral blood defined by *TCR* gene rearrangement, however, is not limited to neoplastic disease processes and has been observed in cases with benign dermatoses such as psoriasis³⁵ and benign exfoliative erythrodermas.³⁶

The recommendations for the interpretation of clonal *TCR* gene rearrangement in the peripheral blood in SS are not clearly defined. The ISCL recommends a combination of increased lymphocyte counts with evidence of a T-cell clone in the blood as a criterion for SS.²¹ The World Health Organization–European Organization for Research and Treatment of Cancer recommends considering the presence of a T-cell clone in the blood only in combination with at least one of the cytomorphic or immunophenotypic criteria to exclude patients with benign inflammatory conditions simulating SS.³⁷

In our study, clonal *TCR* rearrangement was observed in 2 of the benign dermatosis cases. One of these two patients had generalized pruritus without discrete skin lesions and with an extensive history of atopy. The second patient had lymphomatoid papulosis of the leg, diagnosed with repeated skin biopsies. Neither of the two patients had lymphocytosis, an increase in convoluted lymphocytes, or phenotypic aberrancies detected by FCIP of peripheral blood samples. Clonal and oligoclonal lymphocyte expansion has been described in atopic patients,³⁸ and low-level trafficking of clonal T cells through blood vessels has been demonstrated even when a disease seems confined to the skin clinically, such as in lymphomatoid papulosis.³⁹ The presence of a clonal *TCR* gene rearrangement in 2 of 7 benign dermatosis cases seems high and probably represents a sampling bias owing to the small number of patients in the group.

We correlated the percentage of CD26 negativity with the extent of major T-cell antigen loss. The percentage of CD26– cases was low in the range of 0% to 30% T-cell antigen loss and showed a considerable increase beyond 30% T-cell antigen loss. Unlike the percentage of SS cases (as shown in Figure 1B), the percentage of CD26– cases did not parallel the increasing extent of T-cell antigen loss in the range of 40% and beyond but remained relatively stable at about 60%. Clearly, the down-regulation of a major T-cell antigen and the loss of CD26 expression are regulated independently in neoplastic T cells, although they were colocalized in the same T-cell population in 45% of cases.

In our experience, up to 40% of SS cases retain their CD26 positive staining in the peripheral blood as shown by FCIP. In addition, individual patients can show consistently CD26+ results in repeated FCIP tests of the peripheral blood over several years despite fluctuating tumor burden ■**Figure 4A**■. In these patients, the aberrancies of major T-cell antigen expression remain the most useful tool to identify the neoplastic T cells. Considering that the majority of the patients with SS in this study were previously treated (23 of 28), our results

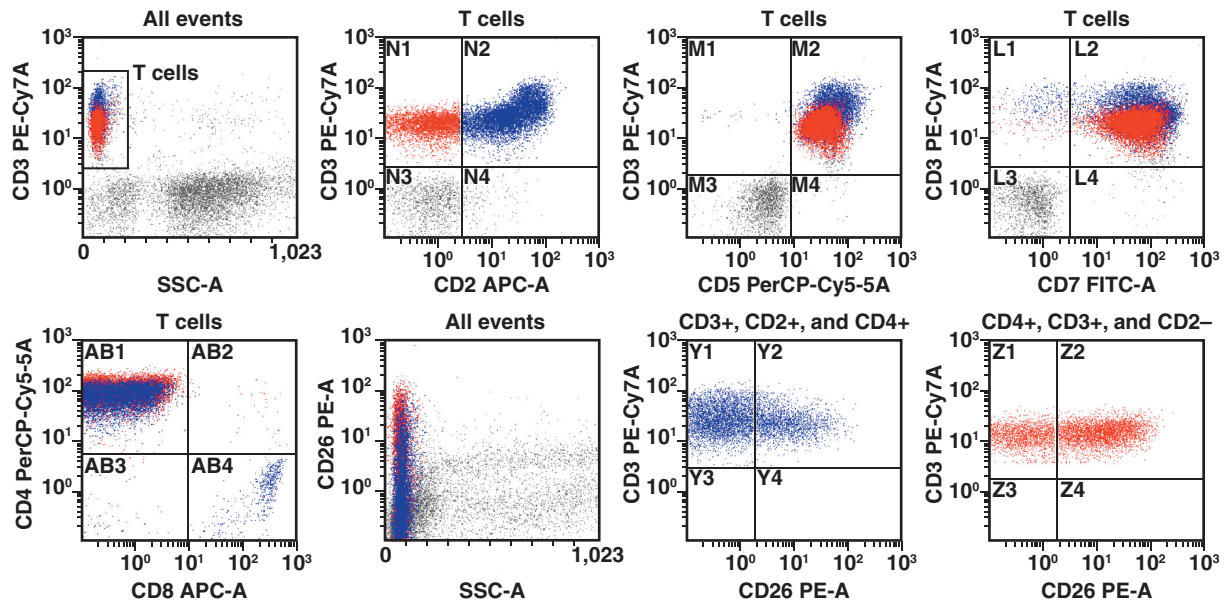
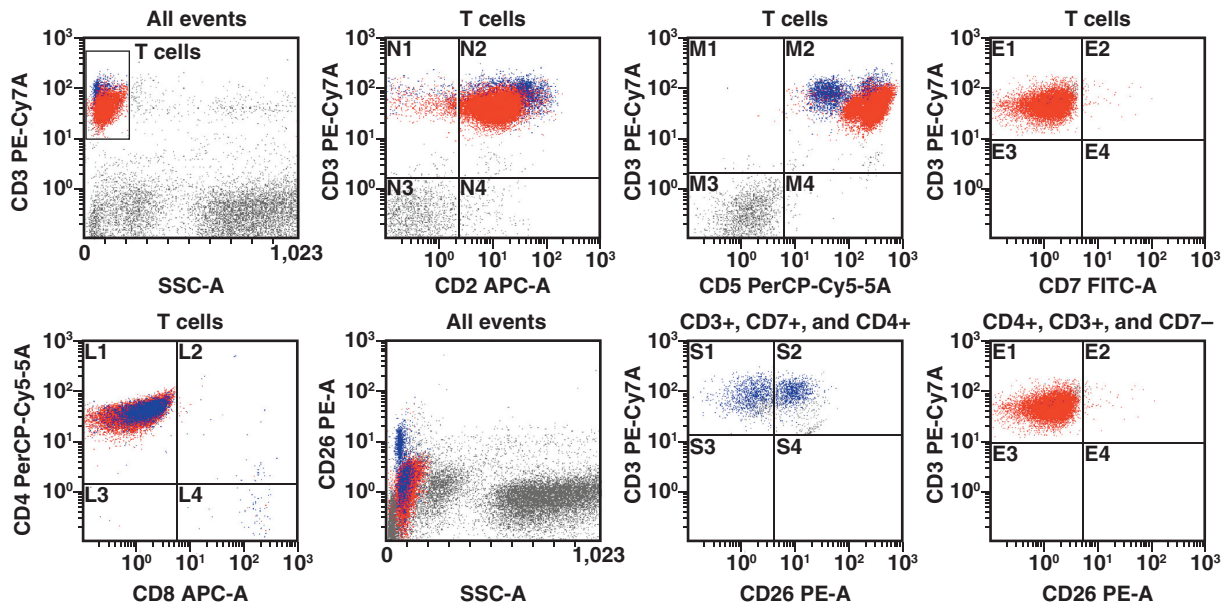
A

B


Figure 4 Different patterns of CD26 expression in patients with Sézary syndrome. **A**, Flow cytometric immunophenotyping (FCIP) of a peripheral blood sample from a 77-year-old man. On the day of analysis, the patient had a WBC count of 13,500/ μ L (13.5×10^9 /L, an absolute number of Sézary cells of 2,633/ μ L of blood, and a CD4/CD8 ratio of 49. There is a distinct T-cell population with down-regulation of CD2 expression, while CD5 and CD7 expression are preserved. CD26 is dim positive ("smear") on all CD4+ T cells. This patient had consistent dim CD26+ results in 8 different FCIP analyses during a period of 18 months with considerably fluctuating tumor burden, which was paralleled by a fluctuating extent of CD2 loss. **B**, FCIP of a peripheral blood sample from an 82-year-old man with a WBC count of 22,000/ μ L (22×10^9 /L, an absolute Sézary cell count of 4,100/ μ L, and a CD4/CD8 ratio of 99. There are 2 distinct T cell populations present. The majority of the T cells are CD3+, CD4+, and bright CD5+ and show down-regulation of CD7 and loss of CD26 expression, which is colocalized with the loss of CD7. A minor CD4+ T-cell population shows brighter CD3 expression and dimmer CD5 staining compared with the rest of the T cells and retains dim CD7 and dim CD26 expression.

C

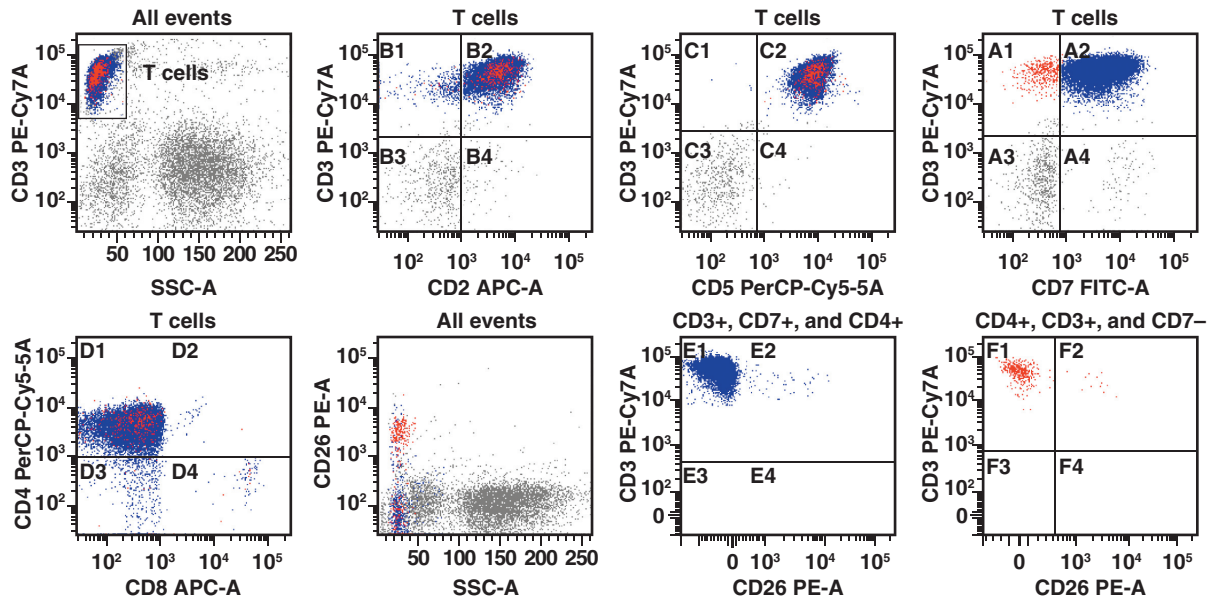


Figure 4 (continued) **C**, FCIP of a peripheral blood sample from a 61-year-old man with a WBC count of $43,000$ ($43 \times 10^9/L$), an absolute Sézary cell count of $24,300/\mu L$, and a CD4/CD8 ratio of 98. There is no major T-cell antigen loss; CD26, however, is lost on all CD4+ T cells. APC, allophycocyanin; FITC, fluorescein isothiocyanate; PE, phycoerythrin; PerCP, peridinin chlorophyll protein; SSC, side scatter.

reflect the usefulness of the studied markers to correctly identify Sézary cells in the peripheral blood, rather than their usefulness for establishing a primary diagnosis of SS. Patients with SS usually require several rounds of treatment and thorough follow-up to monitor the changes of tumor burden. Because SS is a rare condition and flow cytometric laboratories might encounter more peripheral blood samples from treated SS patients than diagnostic specimens, it becomes important to define the usefulness of CD26 in this patient population.

Our data suggest that the sensitivity of CD26– results in SS seems lower than previously suggested and is about the same as the sensitivity of major T-cell antigen loss when a threshold of 40% is used (59%) but considerably lower than a threshold of 30% loss of T-cell antigen could provide (72%). Although the specificity of the CD26– results is 100% with regard to control cases, its specificity is inferior to the T-cell antigen loss with regard to MF and benign dermatosis. FCIP of the peripheral blood remains an effective method to help in the identification of neoplastic cells in SS. Although neither major T-cell antigen aberrancies nor CD26 negativity is highly sensitive alone, an increase of sensitivity can be achieved by combining the diagnostic power of the two approaches (86%) with little loss in specificity.

From the Departments of ¹Pathology, ²Dermatology, and ³Medicine, Northwestern Memorial Hospital, Northwestern University Feinberg School of Medicine, Chicago, IL.

Address reprint requests to Dr Kelemen: Dept of Pathology, Northwestern Memorial Hospital, 251 E Huron, Feinberg 7-205, Chicago, IL 60611.

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