Absence of CD26 Expression Is a Useful Marker for Diagnosis of T-Cell Lymphoma in Peripheral Blood

Dan Jones, MD, PhD,1 Nam H. Dang, MD, PhD,2 Madeleine Duvic, MD,3 LaBaron T. Washington, MD,1 and Yang O. Huh, MD1

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Abstract

We report flow cytometric characterization of surface CD26 expression in 271 peripheral blood samples from 154 patients evaluated for the presence of a T-cell lymphoproliferative disorder, primarily mycosis fungoides/Sézary syndrome (MF/SS). The presence of morphologically identifiable tumor cells on peripheral blood smears was the criterion for lymphomatous involvement. In 66 of 69 samples from 28 patients, we identified an abnormal CD26–/dim T-cell population that was distinct from the variable CD26 expression seen in normal peripheral blood T cells. This population was CD26– in 23 patients and weakly CD26+ in 5 patients. CD7 was more variably expressed in MF/SS tumor cells, allowing recognition of a distinct, quantifiable abnormal T-cell population in only 34 of 69 involved samples. An increased CD4/CD8 ratio and lower surface expression of CD4 in tumor cells also helped separate the CD26–/dim atypical population for quantification. In 35 blood samples from other types of T-cell tumors, tumor cells in 10 of 11 morphologically involved cases showed absent/dim CD26. Although capable of detecting abnormalities in most cases of MF/SS, CD7 expression does not provide as clear a separation of the neoplastic population and can be replaced by CD26 staining in routine peripheral blood flow cytometric screening of MF/SS patients.

Mycosis fungoides (MF) is a cutaneous lymphoma composed of CD4+ T cells with a skin-homing phenotype. A related disorder, Sézary syndrome (SS), is characterized by manifestation as a diffuse erythrodermic rash with substantial numbers of circulating tumor cells. A subset of patients with typical MF also develop SS when the disease progresses. The presence of peripheral blood involvement in MF is a negative prognostic factor.1,2 Molecular studies have detected evidence of clonal T cells in the peripheral blood in many patients with MF, likely representing the circulation of small numbers of tumor cells.2,5 However, the clinical significance of such low-level peripheral blood involvement, detected only by polymerase chain reaction analysis, is unclear given the presence of such clones in patients with clinically localized cutaneous disease.6

MF/SS in blood has a variety of morphologic appearances, including small irregular cells, markedly convoluted classic “Sézary” lymphocytes, and larger nucleolated forms. Because of this variable appearance, accurate enumeration of tumor cells by manual differential can be difficult. For these reasons, flow cytometric analysis is used routinely in many centers to identify circulating MF cells in peripheral blood. Diagnostic criteria have included absolute increases in CD4+ T cells, an elevation of the CD4/CD8 ratio, and increases in the number of CD7– T cells.7-11 However, all of these parameters also can be increased in the peripheral blood in nonneoplastic conditions, including inflammatory dermatoses, hindering the definitive diagnosis of MF/SS or other T-cell tumors.8,9,11 CD26 is a well-characterized surface proteolytic enzyme that is expressed in the majority of lymphocytes in peripheral blood.12 We report that absent or dim CD26 expression is a feature of almost all cases of MF and can be used to allow more sensitive flow cytometric detection and quantitation of tumors cells than the use of CD7.
Materials and Methods

We analyzed 271 consecutive peripheral blood samples (from 154 patients) submitted for evaluation of involvement by a T-cell lymphoproliferative disorder to the immunology laboratory at the University of Texas–M.D. Anderson Cancer Center, Houston, during a 5-month study period (4 months continuous with 1 subsequent month of follow-up). In 83% of the cases, correlation was made with the morphologic findings in peripheral blood smears or bone marrow aspirates prepared from a sample from the same day. For the remaining cases, comparison was made with peripheral blood smears from other dates, usually within a week of the analyzed cytometry sample. Lymphoma cells, defined as either Sézary cells with markedly convoluted nuclear contours or enlarged (nucleolated) lymphocytes, were quantitated by 100-cell manual differential by one of us (D.J.). Serial samples from the same patient were examined, when available, to establish the characteristic morphologic features of each patient’s tumor.

Diagnoses of patients were MF (94), SS (11, 1 in remission), nodal peripheral T-cell lymphoma (12), cutaneous anaplastic large cell lymphoma (3), cutaneous T-cell lymphoma, not otherwise specified (6), CD8+ leukemia/lymphoma (2), natural killer cell lymphoma (2), large granular lymphocyte (LGL) leukemia (2), atypical cutaneous infiltrate/rule out MF (10), and chronic dermatitis (12). Final diagnoses in all cases were based on overall morphologic and immunophenotypic findings in bone marrow, blood, and tissue samples using criteria from the updated World Health Organization classification.13 Cases with primary skin involvement by a CD4+, epidermotropic T-cell tumor were diagnosed as MF or as Sézary leukemia if there was an erythrodermic rash and substantial peripheral blood involvement (tumor cells >500/µL). The diagnosis of “atypical cutaneous lymphoid infiltrate” was used for cases with clinical features suggestive of MF and/or the presence of a clonal T-cell receptor rearrangement by molecular analysis but equivocal histologic findings. At our institution during this period, we typically performed analysis of peripheral blood samples for all patients with systemic T-cell lymphoma and patients with cutaneous findings suggestive of MF.

Three-color flow cytometric analysis was performed on samples following a standard cell lysis method using mouse monoclonal antibodies directed against CD45 (peridinin chlorophyll-a-protein [PerCP]-conjugated), as well as various combinations of CD26 (fluorescein isothiocyanate [FITC]-conjugated, IgG2a clone L272), CD3 (FITC or phycoerythrin [PE]), CD4 (PE), CD5 (PE), CD7 (FITC), CD8 (FITC or PE), and CD19. All antibodies were from Becton Dickinson, San Jose, CA, unless otherwise stated, and analysis was performed using FACSscan or FACS caliber cytometers (Becton Dickinson). Ten control samples from healthy blood donors were used to establish the range of CD26 and CD7 expression in T-cell populations. In cases other than MF, additional antibodies (particularly CD56, CD57, and T-cell receptor-gamma/delta) were used in 4-color analysis to determine the final diagnosis. Negative staining levels were set by comparison with an isotype control antibody. Statistical analysis was performed using the Fisher exact and the Student t tests and Pearson correlation coefficient measures with Stats Direct Software (Camcode, Ashwell, England).

For all samples, analysis was limited to lymphocytes by using a manual gating strategy based on forward/side scatter and side scatter/CD45-PerCP staining properties. Contamination of the lymphocyte gate by monocytes was assessed by CD45-PerCP/CD14-PE staining. The CD26 and CD7 staining patterns were analyzed by eye using the CD4-PE/CD26-FITC, CD3-PE/CD26-FITC, CD4-PE/CD7-FITC, and CD3-PE/CD7-FITC dot-plot cytograms. For some cases, cluster analysis was performed using CellQuest and Paint-a-Gate software (Becton Dickinson). Criteria for an abnormal CD7 or CD26 tumor population included the presence of a CD4+ T-cell population that was clearly separable from the normal spectrum of variably positive reactive lymphocytes.

Results

CD26 and CD7 Expression in Normal Peripheral Blood Lymphocytes

In peripheral blood from 10 healthy control subjects, there was a spectrum of CD26 expression in both CD4+ and CD8+ T cells Image 1A. As shown on the CD4/CD26 cytograms, CD26 positivity ranged between 56% and 86% of the CD3+CD4+ T-cell population (mean ± SD, 77% ± 8%). Normal CD8+ lymphocytes showed similar variable surface CD26 expression, with a range of 24% to 80% showing positive staining (mean ± SD, 52% ± 13% [not shown]). CD3+CD56+ T cells also showed variable CD26 expression (range, 25%-90%) but CD56+CD3– natural killer cells were typically CD26– (up to 10% could be weakly positive). Variation in the percentage of CD26+ peripheral blood T cells in different samples likely was affected by the degree of activation of the normal lymphocyte populations, a characteristic feature of CD26 expression in mature T cells.14 CD7 expression in nonneoplastic T cells was assessed in 19 normal control samples and showed variable positivity of both CD4+ and CD8+ T cells (Image 1A). CD7 expression was seen in 73% to 97% of CD4+CD3+ T cells (mean ± SD, 85% ± 6%) in the samples from healthy control subjects. CD7 expression was more variable on CD4+ T cells in study patients than in control subjects, ranging from 61% to 89%
of CD4+CD3+ cells in samples that showed no morphologic evidence of tumor. These findings suggest that the effects of treatment, reactive T-cell populations, and concurrent illnesses (e.g., infection) may increase the number of circulating CD7– T cells in the absence of circulating tumor.

**CD26 and CD7 Expression in MF/SS**

Overall, CD26 expression was assessed in 271 samples from 154 patients undergoing initial workup or follow-up for a T-cell lymphoproliferative disorder, mostly cutaneous T-cell lymphoma. Comparison was made with morphologic examination of peripheral blood smears done around the same time. In all but 3 of the morphologically uninvolved blood samples, CD26 expression in T cells showed the typical continuous, “straight-line” staining pattern on the CD4-PE/CD26-FITC cytograms. We noted 3 morphologically normal peripheral blood samples (2 from patients with MF, 1 from a patient with an atypical cutaneous T-cell infiltrate) that showed small discrete separable CD4-dim CD26– populations, representing 0.3% to 0.5% of all analyzed cells (data not shown).

Examination of 69 samples from 28 patients with MF or SS with tumor cells in a smear revealed an abnormal population of T cells on either the CD4/CD26 or the CD4/CD7 cytograms in 66 samples (96%). In 23 of these patients, this population was uniformly CD26– and formed a discrete population separable from the variable CD26 staining of benign T cells. In the other 5 patients, the

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**Image 1** CD26 and CD7 expression in peripheral blood T lymphocytes. **A**, Peripheral blood from a healthy control subject demonstrates variable expression of both CD7 and CD26 in CD4+ T cells. **B**, Sézary leukemia. Tumor cells demonstrate uniform absence of expression of CD7 and CD26. **C**, Mycosis fungoides in blood. Dim surface expression of CD4 in tumor cells allows a quantification of clearly separable CD26– and CD7– populations, representing approximately 15% of total events analyzed (21% of the lymphocyte gate). FITC, fluorescein isothiocyanate; PE, phycoerythrin.
abnormal population was weakly CD26+ but was separable from the normal T-cell population because of lower surface expression of CD4. Only 3 morphologically atypical blood samples (all from patients in the patch/plaque stage of MF) did not show a discrete abnormal population on the CD4/CD26 (or CD3/CD26) cytograms. Two samples were involved minimally, showing 1% and 3% identifiable tumor cells, respectively, on manual differential counts of the smear. The remaining smear had rare lymphocytes (<0.1% of cells) suggestive of Sézary cells. Comparing MF/SS, dermatitis, and atypical cutaneous lymphoid lesions, there was a strong association between the identification of a separable CD26–/dim population and identifiable tumor cells on the peripheral smear ($P < .0001$).

In contrast, CD7 was expressed more variably on MF/SS tumor cells. Overall, 34 (49%) of 69 involved blood samples from 28 patients with MF/SS had a separable, quantifiable abnormal T-cell population identified by examination of the CD4/CD7 cytograms. In the remaining cases, it was not possible to separate the abnormal population from the nonneoplastic lymphocytes because of variability in CD7 staining. The existence of an abnormal (neoplastic) population in these cases usually could be inferred (but not quantified) by relative increases in the number of CD7+ T cells. CD4/CD26 dual staining allowed separation of the abnormal T-cell population in all of these cases. There was a single sample in which an abnormal population detected by examination of CD4/CD7 cytograms was not clearly seen by CD4/CD26 staining. Overall, 10 of 28 patients with MF/SS showed abnormal (tumor) T-cell populations that were variably or uniformly CD7+. In 3 patients, there were variations in the expression of CD7 in the presumed tumor cells between samples obtained on different dates.

Serial analyses of peripheral blood samples from patients with MF/SS undergoing treatment showed variations in the numbers of tumor cells counted on smears that paralleled variations in the size of the abnormal population detected by CD4/CD26 surface staining. Especially in tumors undergoing therapy, morphologic counts tended to underestimate the percentages of tumor cells present compared with the flow cytometric detection. However, large changes in the number of the abnormal CD26–/dim T-cell populations detected by the flow cytometric method were paralleled by the findings on the smears. Thus, the CD26– population likely represents the neoplastic population in MF/SS.

### Expression of Other T-Cell Markers in MF/SS

Other flow cytometric features seen in involved samples from patients with MF/SS are summarized in Table 1. Analysis of the CD3+ T-cell population revealed a CD4/CD8 ratio greater than 5 in at least 1 sample from 23 of 28 patients with MF/SS but in only 3 of 139 uninvolved samples ($P < .0001$). This ratio was elevated more frequently in untreated patients and was less often elevated in patients with MF/SS undergoing systemic therapy. It therefore was less useful for detecting low-level lymphomatous involvement.

Lower level expression of CD4 (compared with the level on nonneoplastic T cells) was seen on tumor cells from 14 of 28 patients (Image 1C). In 3 cases showed complete absence of CD4 on some tumor cells. In 2 of the cases, correlation of the results of skin biopsy findings with cluster analysis of the peripheral blood flow cytometric data revealed that this absence of CD4 was associated with the transformed large cell population within the tumor (other data not shown). CD7 staining in these cases was confusing to interpret since variable staining of different
tumor cell populations overlapped with normal CD7 staining (Image 3D). Dim expression of CD5 was seen in tumor cells from 4 of 26 patients (not shown). Tumor cells in 5 patients with MF/SS showed differences in the expression level of CD3 compared with their normal T-cell populations (Image 1C). In all cases, the aberrant level of surface expression of pan T-cell markers was maintained over sequential tumor samples during the 4-month study period, providing a stable “signature” of the tumor immunophenotype (not shown).

Flow Cytometric Detection of CD26 in Other Types of T-Cell Lymphoproliferative Disorders

The peripheral blood findings in a small number of samples from patients with other T-cell tumors are summarized in Table 1. Eleven samples from 7 patients were involved by morphologic criteria; 10 of 11 of these samples showed an abnormal CD26– population on the CD4/CD26 cytograms, with the remaining case showing an abnormal CD26+ population. In the 4 samples from CD4+ peripheral T-cell lymphoma, dim CD4 expression was noted in 2, and dim CD5 and a high level of expression of surface CD3 were noted, respectively, in the other 2 samples, allowing easier recognition of the neoplastic population. Of 7 samples analyzed from 3 CD8+ tumors (LGL leukemia, hepatosplenic lymphoma, and 1 unclassifiable tumor), 6 showed uniform absence of CD26 expression in the tumor cell population. One LGL leukemia was CD26+.

Discussion

Unequivocal detection of neoplastic T-cell populations by flow cytometric analysis is difficult, given the lack of easily applicable methods for demonstration of clonality. Nevertheless, clinicopathologically defined subtypes of T-cell tumors show characteristic immunophenotypic features that can be used to identify them by multiparameter analysis.13,15 The definitive detection of MF cells is limited by the absence of specific immunophenotypic markers for this tumor type. The characteristic MF immunophenotype includes positivity for CD4, CD15s, cutaneous lymphocyte antigen (CLA), CD60, and CXCR3 and absence of expression of CD7 and CD49d (VLA-1 integrin).9,16-18 However, all of these markers also are variably present in normal T-cell populations, making quantification of tumor involvement
difficult. We showed that absence of expression of CD26 also is highly characteristic of MF/SS tumor cells and that an abnormal CD26− population correlates with the presence of morphologically identifiable tumor cells in the peripheral blood (96% sensitivity, 98% specificity).

The best-studied MF-associated marker is CLA, an adhesion molecule that mediates the cutaneous localization of skin-homing lymphocytes. CLA is expressed in most skin-homing T cells and almost all MF tumor cells within skin, as well as in a small subset of circulating benign lymphocytes, especially in patients with chronic dermatitis. However, MF/cutaneous T-cell lymphoma (CTCL) tumor cells tend to shed CLA expression outside the skin microenvironment, and there is not a good correlation between the number of CLA-positive cells in the blood and the number of Sézary cells counted or the degree of lymph node involvement in MF. Molecular analysis of sorted cell populations has demonstrated that circulating MF tumor cells are present in both the CLA-positive and the CLA-negative populations. This result is supported by the lability of CLA expression noted on T cells in culture. Thus, CLA is unlikely to be a highly sensitive antigen for detection of circulating MF cells.

The most commonly used method for detection of cutaneous lymphoma circulating in peripheral blood is the absence of CD7 expression on CD4+ T cells. In normal T
cells, CD7 is low or absent in most skin-homing lymphocytes and in a variable number of circulating T cells. It has been reported that the size of the CD7− T-cell population correlates with the extent of disease in MF/CTCL. However, as described, approximately 50% of MF/CTCL cases show variable CD7 expression, making tumor cells difficult to distinguish from normal and nonneoplastic reactive T-cell populations. We found that unless the MF tumor cells show lower surface expression of CD4 (or CD3 or CD5), it is difficult to separate them definitively from normal T cells by cluster analysis with CD7 staining. In contrast, the reproducible low or negative expression of CD26 in MF usually creates a separable discrete cluster of tumor cells that can be recognized.

CD26 (also known as dipeptidyl peptidase IV) is a widely expressed cell surface proteolytic enzyme related to cellular activation that is expressed in the majority of circulating T cells. As shown herein, CD26 is expressed variably in most CD4+ circulating T cells, and it is expressed at highest levels on the activated memory-helper T-cell subset, but also is expressed at varying levels in CD8+ and immature T-cell populations. Absence of CD26 expression has been reported in small numbers of other T-cell tumor types, including adult T-cell leukemia/lymphoma and MF in skin. Whether CD26 will prove useful for the detection of T-cell tumor cells other than MF/SS requires further study. In a small number of cases, we noted absence of CD26 expression in blood involvement in 5 patients with peripheral T-cell lymphoma and 3 patients with CD8+ tumors. Previously, small studies have demonstrated that CD26 expression is associated with certain tumor types—anaplastic large cell lymphoma, precursor T-lymphoblastic lymphoma, and hepatosplenic lymphoma—that we did not study. Our study supports the use of CD26, in place of or in addition to CD7, as part of the routine panel for flow cytometric detection of neoplastic T cells in MF/SS.

From the Division of Pathology and Laboratory Medicine and the Departments of Dermatology, the University of Texas—M.D. Anderson Cancer Center, Houston.

Address reprint requests to Dr Jones: Dept of Hematopathology, Box 72, 1515 Holcombe Blvd, Houston TX 77030.

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