Immunophenotypic Analysis of Peripheral T-Cell Neoplasms

A Multiparameter Flow Cytometric Approach

Saba Jamal, MD,* Louis J. Picker, MD,* Deborah B. Aquino, MD, Robert W. McKenna, MD, D. Brian Dawson, PhD, and Steven H. Kroft, MD

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Abstract

We retrospectively reviewed multiparameter flow cytometric analyses in 50 peripheral T-cell neoplasms (PTCNs). Results were interpreted within the context of a large cohort of nonneoplastic T-cell populations. All PTCN diagnoses were confirmed with morphologic and/or molecular analysis. Aberrant populations were defined as discrete immunophenotypic clusters exhibiting loss of or increased or diminished expression of T-cell antigens relative to internal immunophenotypically normal T-cell populations. An antigenic pattern was considered abnormal if it exceeded ranges for T-cell subsets in specific anatomic sites or was not normally encountered. Forty-six of 50 and 41 of 50 demonstrated 1 or more and 2 or more aberrations, respectively. The most common abnormally expressed antigen was CD3, followed by CD7, CD5, and CD2. Except for CD7, abnormally dim or bright antigen expression was more common than deletion. Only 3 cases were abnormal solely based on expansion of an otherwise immunophenotypically normal population; the remainder had patterns of antigen expression not seen in nonneoplastic populations. These data indicate that most PTCNs are aberrant by multiparameter flow analysis. However, results must be interpreted within the context of thorough knowledge of the immunophenotypic spectrum of nonneoplastic T cells.

Peripheral T-cell neoplasms (PTCNs) are a diverse group of tumors derived from postthymic T cells. They have a wide morphologic spectrum, which at times overlaps with reactive lymphoid proliferations. Demonstration of the neoplastic nature of T-cell proliferations traditionally has relied on morphologic criteria, although more recently molecular techniques, particularly polymerase chain reaction (PCR), have assumed a central role. However, PCR techniques are not entirely sensitive or specific. Commonly used primer sets do not hybridize with all possible rearrangements. Conversely, clinically benign T-cell proliferations may contain clonal bands by PCR analysis.

Immunophenotypic assays have generally been used in PTCNs to confirm the T-cell nature of an atypical infiltrate, rather than to establish clonality, as there are no generally used, reliable immunophenotypic markers of T-cell clonality. Analysis of V-beta gene use by flow cytometry has been used to assess clonality of T-cell populations, but this technique is cumbersome and is little used outside research settings. The majority of immunophenotypic studies of PTCNs have used single-parameter techniques such as frozen- or paraffin-section immunoperoxidase or indirect immunofluorescence studies on cell suspensions. Traditional criteria for aberrancy have included T-cell predominance, T-cell subset restriction, gross antigen deletion, expression of antigens not normally present on T cells, and coexpression or loss of both CD4 and CD8. Such techniques are unable to reliably detect subtle changes in antigen expression. In addition, many PTCNs contain a prominent reactive T-cell infiltrate that is difficult to distinguish from the neoplastic populations.

Multiparameter flow cytometry (MPFC) has the potential for increased specificity and sensitivity compared with...
single-parameter immunophenotypic techniques in the diagnosis of PTCL. This technique permits the independent assessment of the antigenic profiles of various T-cell populations within a tissue sample, even very small ones. In addition, it permits detection of subtle alterations in antigen expression, rather than just gross deletions. Importantly, such alterations result in novel cell populations in fluorescence histograms (dot plots) that may be discriminated from internal T-cell populations with normal antigen expression (cluster analysis). However, interpretation of the significance of these populations is complicated by the complexity of the T-cell immune response. In addition to major T-cell subsets, a variety of small normal subsets defined by patterns of antigen expression have been described. These may vary in size, depending on anatomic site, and expand in various disease states. Furthermore, T cells have been shown to up-regulate or down-regulate expression of certain antigens in certain disease states and in vitro settings.

These latter changes are important in the diagnostic setting only if they produce populations forming clusters in MPFC histograms that are distinct from other T-cell populations within a sample.

In this article, we review our institutional experience with flow cytometric assessment of PTCNs in order to determine the frequency and spectrum of immunophenotypic aberrancy and whether it is a useful technique in the diagnosis of these tumors. To facilitate the analysis of neoplastic T-cell processes, we established the spectrum of antigen expression in various T-cell subsets in different anatomic sites. By using 3- or 4-color flow cytometry with cluster analysis, we established reference ranges for known nonneoplastic T-cell subsets in several anatomic sites for our patient population (encompassing a wide range of disease states). In addition, we catalogued the nature and frequency of any additional T-cell populations with unusual patterns of antigen expression. These data then can function as a baseline for the assessment of immunophenotypic aberrancy in suspected T-cell neoplasms.

Materials and Methods

Nonneoplastic T-Cell Populations

To establish a baseline for assessing aberrant T-cell populations, ranges of various T-cell subsets in specific anatomic sites were defined in our patient population. A retrospective review of flow cytometry samples was performed during 1998 of consecutive blood, bone marrow, and lymph node samples during 8-, 1-, and 5-month periods, respectively. Spleen and bowel samples were accrued from March 1994 through January 1999. Any cases with pathologic evidence of T-cell neoplasia were excluded. Ultimately included were 126 bone marrow, 100 blood, 99 lymph node, 24 spleen, and 11 small bowel samples. All blood, bone marrow, and lymph node specimens were analyzed with 4-color flow cytometry, whereas 7 of 24 spleen samples and 3 of 11 bowel samples were analyzed with 3-color flow cytometry. A variety of antibody panels are used in our laboratory depending on the source of the specimen and the indication for the flow cytometric analysis. In turn, the combinations of T-cell–associated antibodies used varies between panels.

Three-color combinations (fluorescein isothiocyanate [FITC], phycoerythrin [PE], peridinin-chlorophyll [PerCP]) included the following: CD7/CD4/CD8, CD2/CD56/CD3, CD5/CD19/CD3 (for most tissue, blood, and fluid specimens); CD7/CD56/CD3, CD5/CD4/CD8 (for acute leukemias in blood or bone marrow); or CD7/CD4/CD8, CD5/CD19/CD3, CD3, CD45RO, CD45RA (for lymphoma in bone marrow). Four-color combinations included the following (FITC, PE, PerCP, allophycocyanin): CD30/CD2/CD45/CD3, CD7/CD4/CD8/CD45RO, FMC7/CD23/CD5/CD19 (for tissue and fluid specimens); CD2/CD45/CD3/CD45RO, CD7/CD8/CD3, FMC7/CD23/CD5/CD19 (for lymphocytosis and lymphoma in bone marrow); CD8/CD5/CD3/CD4 (for routine marrow screens); CD5/CD4/CD8/CD19 (for plasma cell dyscrasias); CD8/CD5/CD3/CD4, CD7/CD1a/CD3/CD45RO (for pediatric acute leukemia); or CD8/CD5/CD3/CD4, CD61/CD2/CD45/CD34, CD7/CD13/HLA-DR/CD34, intracellular terminal deoxynucleotidyl transferase (TdT)/CD22/CD3/CD45 (for adult acute leukemia). Antibodies used relevant to T cells are as follows (all from Becton Dickinson, San Jose, CA, unless otherwise specified): CD1a (H149), CD2 (55.2), CD3 (SK7), CD4 (SK3), CD5 (L17F12), CD7 (4H9), CD8 (SK1), CD30 (BerH2; DAKO), Carpiniteria, CA), CD45 (2D1), CD45RO (UCHL-1), CD45RA (L48), CD56 (MY31), TdT (HT-1, HT-3, and HT-4; DAKO), T-cell receptor (TCR) alpha/beta (WT31), and TCR gamma/delta (11F2).

The specimens were analyzed for a variety of diagnoses. Table 1 and Table 2; many contained non–T-cell neoplastic processes. The T-cell subsets quantified for all sites were as follows:

- CD4+/CD8– T cells as a percentage of total T cells
- CD4–/CD8+ T cells as a percentage of total T cells
- CD4+/CD8+ T cells as a percentage of total T cells
- CD4–/CD8(dim to –) T cells as a percentage of total T cells
- CD7(dim to –)/CD4+ T cells as a percentage of total CD4+ T cells
- CD7(dim to –)/CD8+ T cells as a percentage of total CD8+ T cells
- CD2– T cells as a percentage of total T cells
- The CD5– subset of CD8+ cells was quantified only for

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peripheral blood and bone marrow samples. It was not possible to quantify this subset in other sites because of the lack of appropriate combinations of antibodies in the panels used for specimens other than blood and bone marrow. CD5-/CD4+ cells were not quantified because of too few events with this profile and lack of clustering (see the next paragraph). In lymph node, spleen, and bowel specimens, anti-CD3 was not included in the same tube as anti-CD4 and anti-CD8 antibodies; rather, the latter 2 antibodies were accompanied by CD45R0 and CD7 in the 4-color panel and CD7 in the 3-color panel. Because of this, the population of CD4−/CD8(dim to −) cells generated from these tubes contained natural killer (NK) cells as well as T cells. To correct for this, the percentage of NK cells (CD2+/CD3−) was determined in a separate tube in each panel (CD30/CD2/CD45/CD3 or CD2/CD56/CD3) and subtracted from the percentage generated in the anti-CD4/CD8−containing tubes.

T-cell subsets were defined as discrete “clusters” in 1 or more of the 2-dimensional histograms derived from 4-color or 3-color flow data, with a few exceptions. For example, a continuous spectrum from negative to positive often is observed in the CD7 expression on T cells. When 2 discrete clusters were evident, this was used as the dividing point. However, when populations were continuous, the cutoff was defined by comparison with appropriate isotypic controls. In addition, CD4/CD8 dual-negative cells often show continuity with dim CD8+ cells. The dual-negative/dim CD8+ cells are known to primarily represent a single functional subset (gamma/delta T cells). Therefore, for simplification of analysis, dim CD8+ cells were included in the CD4/CD8 dual-negative subset. Cases also were screened for any populations with negative, dim, or bright expression of CD3; distinct populations of CD30+ T-cells; populations with dim CD4, CD5, or CD45; and populations with bright CD4, CD8, CD5, CD7, and CD45. Care was taken to analyze only viable cellular events based on light scatter properties, as nonviable cells may form artifactual clusters with unusual patterns of antigen expression (eg, dim CD3).

### Peripheral T-Cell Neoplasms

Multiple institutional databases within the University of Texas Southwestern system (including the Diagnostic Molecular Pathology Laboratory) were searched retrospectively from January 1993 to September 1998 for all peripheral T-cell neoplasms for which MPFC had been performed. In addition, cases submitted with a suspicion of T-cell lymphoma also were retrieved. Cases of lymphoproliferative disorder of large granular lymphocytes and anaplastic large cell lymphoma were excluded. The malignant nature of the T-cell proliferation was established in all cases by morphologic review, with or without molecular confirmation. This yielded 50 PTCNs.

### Immunophenotypic Analysis

PTCNs were analyzed over a period of 5 years and 8 months with multiple methods. Fresh tissue was processed in a variety of ways depending on the source of the tissue and the period during which it was analyzed, as detailed elsewhere. Two-color analysis (6 cases) was performed on a Coulter Profile flow cytometer (Coulter, Miami, FL); 3-color analysis (24 cases) was performed on a FACSort flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA); and 4-color analysis (20 cases) was performed on a FACSCalibur flow cytometer (Becton Dickinson). Data analysis for 3- and 4-color flow cytometry was performed using Paint-A-Gate software (Becton Dickinson). Antibodies used in analysis included anti-CD3, -CD4, -CD8, -CD5, -CD7, -CD2, and -CD45. Various combinations of T-cell–associated antibodies are detailed in the section on nonneoplastic T-cell populations. It is important to note that the antibody combinations used in the diagnosis of abnormal or unusual T-cell populations were not limited to the aforementioned combinations. In the face of an abnormality, various custom combinations of antibodies were used routinely to fully characterize an unusual population. These varied from case to case, based on the characteristics of the population in question, and will not be discussed in detail here.

Immunophenotypic aberrations were defined in 2 ways: (1) expansion of a subset beyond site-specific upper ranges determined in our population and (2) a pattern of antigen...
expression not seen in our survey of nonneoplastic T-cell populations. The latter required the presence of novel cell populations forming discrete clusters with abnormal antigen expression relative to internal normal populations. The assessment of dim or bright expression of an antigen in a population was qualitative, based on a definite shift of that population cluster compared with internal normal population clusters. Note that the internal normal T-cell populations could be identified definitively in all cases in which they were present based on the overall pattern of antigen expression, as assessed with appropriate antibody combinations. As a simple example, consider the situation in which 2 distinct populations of T cells are present in a sample based on differing CD3 intensities. If CD4 and CD8 are assessed in the same tube as CD3, one population would consist of a mixture of CD4 and CD8 cells, whereas the other would express only one (or neither) of these markers, permitting definitive assignment of 1 population as normal and 1 as abnormal. In the 2 cases in which no normal T cells were present, only gross abnormalities of expression were considered aberrant.

Assessment of antigen abnormality was sometimes dependent on the other immunophenotypic features of the population in question. For example, if a population was known to express the TCR gamma/delta, then lack of CD4 and CD8 and dim or negative CD5 expression were not considered aberrations, per se. However, if the CD4/CD8 dual-negative population in a sample exceeded the site-specific range established for that subset, it was considered an aberrancy, regardless of other immunophenotypic features. As another example, CD7 negativity usually was considered an aberrancy if the proportion of negative cells exceeded the ranges established for the appropriate T-cell subset (eg, CD4+ or CD8+). However, if the population in question was aberrant in another way that clearly distinguished it from the normal T cells remaining in the sample (eg, loss of CD3), CD7 was considered aberrantly negative if the entire abnormal population lacked it, regardless of the size of the neoplastic population.

Molecular Analysis

T-gamma PCR was performed in 43 cases according to a previously described method.  

Results

Nonneoplastic T-Cell Populations

The diagnoses in cases analyzed for nonneoplastic T-cell populations are summarized in Tables 1 and 2. A summary of the ranges, medians, and 95th percentiles for each T-cell subset in each site is given in Table 3. Details for qualitatively outlying cases for each subset are listed as footnotes; 9 of 12 outliers occurred in association with non-T-cell neoplastic disorders. Details of minor T-cell subsets are provided in Figures 2-3. Examination of light scatter properties revealed that all nonneoplastic T-cell subsets within a given case demonstrated light scatter patterns within the same region (Figures 2 and 3).

CD4/CD8 Dual-Positive Populations

Most commonly encountered was a discrete population of CD5(bright)/CD8(dim) cells (Figure 2A). The next most common pattern was CD4(dim)/CD8(bright) (Figure 2B). Finally, CD4(bright)/CD8(bright) populations were encountered infrequently (Figure 2C).

Normal Antigen Variability

CD8+ cells are characteristically slightly dimmer for CD5 than CD4+ cells (Figure 3E). CD2 is up-regulated on CD45RO+ T cells compared with CD45RO– T cells (Figure 3D). Often producing a bimodal distribution.

Populations With Abnormal CD3 Intensity

These were seen in 8 (8.0%) of 100 blood cases and 6 (4.8%) of 126 bone marrow cases. No populations with abnormal CD3 intensity were seen in lymph node, spleen, or bowel specimens. Thirteen of 14 cases had abnormally bright CD3. Eleven of these 13 populations also were CD2–. CD8+ cells are characteristically slightly dimmer for CD5 than CD4+ cells (Figure 3E). CD2 is up-regulated on CD45RO+ T cells compared with CD45RO– T cells (Figure 3D). Often producing a bimodal distribution.

Of the 14 cases with abnormal CD3 populations, 8 occurred in association with small B-cell lymphoproliferative disorders (chronic lymphocytic leukemia/small lymphocytic lymphoma, 5; follicular lymphoma, 1; hairy cell leukemia, 1; unclassified CD5+ B-cell disorder, 1), 1 with a T-cell–rich large B-cell lymphoma, 2 with AML, 1 with a T-lymphoblastic lymphoma, 1 in an HIV-positive patient, and 1 in a negative study. Overall, B-cell lymphoproliferative disorders were overrepresented in the abnormal CD3 group (P = .006) in blood and marrow. There was also a trend toward overrepresentation of neoplastic disease in general in this group (P = .12).
Figure 1 Examples of neoplastic T-cell populations. In all examples, neoplastic T cells are shown in red and benign T cells in green. A, Angioimmunoblastic T-cell lymphoma. The neoplastic cells have distinctly greater forward light scatter than the normal T cells. They are surface CD3−, have bright intracytoplasmic CD3 compared with the normal T cells, are slightly bright for CD2 (beyond normal biphasic pattern of CD2, which is evident in the nonneoplastic T cells), and lack both CD4 and CD8. B, Unclassifiable peripheral T-cell neoplasm. The neoplastic cells have a greater range of forward light scatter than the normal T cells, show dim CD3 and partial CD2 expression, are predominantly CD7−, and are largely CD4− and CD8− with the exception of a small proportion of cells that are CD4(dim). C, Mycosis fungoides. In this example, the neoplastic cells have the same light scatter distribution as normal T cells. However, they form a distinct CD4(dim) population that is also CD2(dim) and CD5(slightly dim). D, This nodal peripheral T-cell lymphoma, unspecified, has distinct light scatter properties compared with normal T cells. It is CD3(bright), CD4(variable), and CD45(bright). E, This peripheral T-cell lymphoma, unspecified, was morphologically typical of what has been known as Lennert lymphoma. The neoplastic cells have the same light scatter characteristics as normal T cells. However, they are CD3(dim), and the entire population is CD7−. F, The neoplastic cells of this intestinal T-cell lymphoma associated with enteropathy have distinctly greater forward light scatter than normal T cells, have only partial expression of CD3, and are CD2(dim) and CD7(bright). The neoplastic cells are also CD5− and CD4−/CD8−. However, because the cells are T-cell receptor gamma/delta−expressing, these latter 2 findings are not considered aberrant, per se.
Patterns of Antigen Expression Not Encountered

There were no discrete T-cell populations expressing CD30 (only assessed in lymph nodes, spleen, and bowel specimens). No CD3− T cell populations (eg, CD5+/CD4+/CD3−, CD5+/CD8+/CD3−, CD4+/CD7+/CD3−, or CD2+/CD45RO+/CD3−) were encountered in blood or bone marrow samples. Our standard antibody combinations used in lymph nodes, spleen, and bowel specimens would have precluded detection of small numbers of such cells if present. No discrete populations were encountered in any site that was CD2(dim or bright) (beyond the normally observed variability), CD4+/CD5−, CD4dim/CD8−, CD5(bright), CD7(bright), CD8(bright), or CD45(dim or bright).

### T-Cell Neoplasms

The diagnoses of the 50 PTCNs using the Revised European-American classification of lymphoid neoplasms (REAL) classification1 are as follows: peripheral T-cell lymphoma, unspecified, 25 cases (6 composed of small cells or small and medium cells; 19 composed of medium or medium and large cells); mycosis fungoides (MF), 6 cases;
hepatosplenic gamma/delta T-cell lymphoma, 5 cases; lymphoepithelioid (Lennert) lymphoma, 2 cases; intestinal T-cell lymphoma, 1 case; T-prolymphocytic leukemia, 1 case; and unclassifiable PTCN, 7 cases. The unclassifiable cases were considered as such because the diagnoses were made on peripheral blood or bone marrow specimens. The 50 PTCNs occurred in 31 males and 19 females ranging in age from 2 to 86 years (median, 57.5 years). The diagnosis was made on tissue from the following sites: lymph node, 27 cases; bone marrow, 8 cases; blood, 11 cases; spleen, 2 cases; liver and bone, 1 case each. T-gamma PCR was performed in 43 cases and was positive in 34.

**Immunophenotypic Findings**

One to 5 aberrations were found in 46 (92%) of 50 cases **Table 4**. The neoplastic cells in these 46 cases constituted from 7% to 100% of T cells in the sample (median, 59.5%; mean, 56%; SD, 29%) and from 1% to 99% of total cellular events (median, 32.5%; mean, 37%; SD, 26%). The most common aberrant antigen was abnormal CD3 expression (66% of cases) followed by abnormalities in CD7 (58%), CD5 (43%), CD2 (28%), and CD45 (6%) **Table 5**. Examination of Table 5 reveals that, with the exception of CD7, only a minority of aberrant cases completely lacked expression of the various antigens; more common was abnormally dim or bright intensity of antigen expression. The

**Table 4**

Number of Aberrations in 50 Peripheral T-Cell Neoplasms

<table>
<thead>
<tr>
<th>No. of Aberrations</th>
<th>No. (%) of Cases</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>4 (8)</td>
</tr>
<tr>
<td>1</td>
<td>5 (10)</td>
</tr>
<tr>
<td>2</td>
<td>16 (32)</td>
</tr>
<tr>
<td>3</td>
<td>17 (34)</td>
</tr>
<tr>
<td>4</td>
<td>7 (14)</td>
</tr>
<tr>
<td>5</td>
<td>1 (2)</td>
</tr>
</tbody>
</table>

**Table 5**

Frequency of Various Types of Abnormalities as a Percentage of Total Cases (n = 50)*

<table>
<thead>
<tr>
<th>Type of Alteration</th>
<th>CD3</th>
<th>CD7</th>
<th>CD5</th>
<th>CD2</th>
<th>CD45</th>
</tr>
</thead>
<tbody>
<tr>
<td>Overexpression</td>
<td>10</td>
<td>4</td>
<td>24</td>
<td>10</td>
<td>2</td>
</tr>
<tr>
<td>Underexpression</td>
<td>30</td>
<td>18</td>
<td>9</td>
<td>14</td>
<td>4</td>
</tr>
<tr>
<td>Absent expression</td>
<td>26</td>
<td>36</td>
<td>9</td>
<td>4</td>
<td>—</td>
</tr>
<tr>
<td>Total</td>
<td>66</td>
<td>58</td>
<td>42</td>
<td>28</td>
<td>6</td>
</tr>
</tbody>
</table>

* CD5 not assessed in 5 cases (total cases = 45); CD45 not assessed in 2 cases (total cases = 48).
**Figure 3** Other normally encountered T-cell subsets. 


**Figure 4** This histogram illustrates the commonly seen bimodal pattern of CD2 expression. In the CD2 vs CD3 plot, the T-cell population cluster has a bean-shaped distribution. This is a result of greater CD2 intensity on CD45RO+ T cells (primarily memory cells) compared with CD45RO− cells (primarily naive cells).

**Figure 5** Population of CD3(bright)/CD2~ T cells, shown in red; green, other T cells.
immunophenotypically defined lymphoma populations in 16 (35%) of 46 aberrant cases showed light scatter properties distinct from normal lymphocytes in the samples. Representative dot plots of cases with various aberrations are shown in Figure 1.

Of the 46 lymphomas in which an aberrant population was characterized by flow cytometry, 30 (65%) were CD4+, 11 (24%) were CD8+, and 4 (9%) were CD4/CD8 dual negative. No case showed dual expression of CD4 and CD8 on the neoplastic population. However, a single case demonstrated an aberrant T-cell population defined on the basis of dim CD3 expression and lack of CD7 that had distinct CD4+ and CD8+ subsets. This case was clonal by T-gamma PCR. Of the 30 CD4+ cases, 4 (13%) had abnormally dim CD4 expression. Four of 11 CD8+ tumors had dim expression of this antigen. Four PTCNs demonstrated no aberrant populations by flow cytometry. These included 2 peripheral T-cell lymphomas, unspecified, an angioimmunoblastic T-cell lymphoma; and a lymph node overtly involved by MF. Detailed analysis led to the conclusion that the neoplastic cells were not represented in the specimens sent for flow cytometry in the first 2 cases. The angioimmunoblastic T-cell lymphoma, while diagnosed on a morphologic basis by several hematopathologists, lacked

In 12 (46%) of 26 cases in which the neoplastic cells expressed normal intensity CD4, the percentage of CD4+ cells of total T cells exceeded the site-specific ranges previously established and, thus, was considered an aberrancy. One CD4+ tumor occurred as a bone lesion, and site-specific ranges were not available for this case. Of the 7 cases with normal CD8 intensity, 3 had CD8 percentages that exceeded site-specific ranges. Finally, the percentage of CD4+/CD8− cells of total T cells exceeded site-specific ranges for this subset for all 4 CD4/CD8 dual-negative tumors.

Only 3 cases were diagnosed on the basis of expansion of otherwise immunophenotypically normal T-cell populations beyond ranges established in nonneoplastic samples. These included 1 PTCN unspecified with 62% of CD4+ cells in peripheral blood lacking CD7, 1 MF in lymph node with 54% of CD4 cells lacking CD7, and 1 PTCN unspecified with CD4+ cells representing 97% of total T cells in bone marrow. All other cases demonstrated novel cell populations not encountered in samples lacking T-cell neoplasia.

Four PTCNs demonstrated no aberrant populations by flow cytometry. These included 2 peripheral T-cell lymphomas, unspecified, in liver and retroperitoneum; an angioimmunoblastic T-cell lymphoma; and a lymph node overtly involved by MF. Detailed analysis led to the conclusion that the neoplastic cells were not represented in the specimens sent for flow cytometry in the first 2 cases. The angioimmunoblastic T-cell lymphoma, while diagnosed on a morphologic basis by several hematopathologists, lacked...
clusters of clear cells and was negative for TCR gene rearrangements. Furthermore, the patient was alive with no evidence of disease 3.5 years after doxorubicin-based aggressive lymphoma therapy. Therefore, the possibility that this in fact represented an atypical reactive proliferation cannot be ruled out entirely. Finally, the case of MF clearly represented an immunophenotypic false negative, and a subsequent morphologically involved peripheral blood specimen also failed to demonstrate immunophenotypic aberrancy.

Discussion

We have demonstrated in this study the value of MPFC in the characterization of a spectrum of PTCNs. All but 4 of 50 cases demonstrated immunophenotypic aberrancy, a sensitivity of 92%. This exceeds the results of older studies that have demonstrated aberrancy in 17% to 82% of PTCNs. Effort was made to avoid selection bias in favor of immunophenotypically aberrant cases by searching multiple institutional databases (including Diagnostic Molecular Pathology) for cases diagnosed as, or submitted as suspicious for, a PTCN. It remains possible, however, that specimens containing PTCNs received for flow cytometry from outside institutions could have been excluded if they lacked immunophenotypic aberrancy. However, based on the fact that in our practice most discrepancies between flow cytometry results and the morphologic impression are brought to our attention by the referring pathologist (in fact, usually the morphologic preparation is submitted for consultation), we do not believe this was a significant factor in our case selection.

The older immunophenotypic studies with which we have compared our results used single-parameter techniques such as frozen-section or paraffin immunoperoxidase, indirect immunofluorescence, or single-color flow cytometry. Aberrancy in these studies generally entailed gross loss of antigen expression or dual positivity or negativity for CD4 and CD8. In the present study, abnormal intensity of antigen expression was considerably more common than gross deletion for each pan T-cell antigen except CD7. Thus, the increased sensitivity seen in the present study seems to derive from the ability of our flow cytometric technique to detect subtle alterations of antigen expression on specific subpopulations of cells.

Such subtle alterations are best detected with the application of cluster analysis. Instead of drawing lymphoid gates based on scatter and/or CD45 expression and enumerating events exceeding predetermined fluorescence thresholds (quadrant analysis), all events are examined together in dot plots to identify distinct clusters based on any combination of scatter and fluorescence. Distinct clusters usually correspond to distinct cell populations, which can be examined separately for expression of various antigens. In the case of T cells, populations with aberrant antigen expression, including dim or bright expression of normal antigens, may be distinguished from the normal T cells that are present in most cases. Use of “painting” programs (such as Paint-A-Gate) facilitate this process, as each population in a tube can be assigned a different color, which is then applied to all histograms generated from a given tube. In addition to improvements in sensitivity, the approach detailed in the present study also affords improved specificity. Multicolor flow cytometry permits assessment of distinct populations for multiple antigens simultaneously. The lack of specificity in previous analyses is well illustrated in the largest study of the immunophenotypic findings in PTCNs published to date, that of Nakamura et al. In this study, 174 cases were analyzed using several single-parameter techniques in both tissue sections and cell suspensions. More than a third of cases could not be characterized for expression of CD4 or CD8 because of the presence of large numbers of both CD4 and CD8 cells in the specimen.

Of the 46 cases of PTCN that were immunophenotypically aberrant, almost 90% were abnormal in more than one respect, and more than half had at least 3 aberrations. The most frequently abnormal antigen was CD3, which was found to be aberrantly expressed in two thirds of cases. This result is somewhat surprising, given that CD5 and CD7 are usually considered to be the most frequently aberrant antigens in PTCN. The highest reported rates of CD3 abnormality in previous studies have been in those of Nakamura et al and Weiss et al, who described CD3 abnormalities in 37 of 174 cases (21.3%) and 13 of 50 cases (26%), respectively. In the former study, 28 of the 37 cases with CD3 loss were described as “partial.” Of note, the proportion of CD3-deleted cases in these studies correlates well with the percentage of PTCNs in the present study with absent CD3 (26%). Other studies showed CD3 aberrancy in 8% to 17% of cases. Thus, it seems likely that the increased proportion of cases with aberrant CD3 in our study derives from the ability to distinguish populations with altered, but not totally absent, antigen expression. Importantly, 10% of our cases had increased CD3 intensity.

Another potential source of decreased sensitivity in older studies is the difficulty of detecting loss of CD3 in tissue sections when cytoplasmic CD3 is expressed. It also should be noted that we specifically excluded anaplastic large cell lymphomas from our series, as did Hastrup et al; in our experience, these are usually surface CD3− (unpublished observations).

We detected aberrancy in the expression of CD7, CD5, and CD2 in 58%, 42%, and 28% of cases. Literature review reveals aberrancy in these 3 antigens in 45% to 64%, 8,10,15 5%
to 46%, 8,10,11,15,32,33 and 15 to 24%, 8,10,11,15,32 respectively. The source of such wide variability between studies is not readily evident, but it may be a combination of differences in methods and case mix. Our rates of aberrancy for each of these antigens are similar to the highest values reported in the literature. One additional antigen that is infrequently expressed aberrantly, but that we believe is essentially diagnostic of neoplasia, is CD45, which was dimly or brightly expressed in 3 (6%) of 50 cases.

A feature that we found to be quite helpful in approximately one third of the PTCNs we evaluated in the present study was a distinctly different light scatter pattern in 1 T-cell subset compared with others in a sample. In nonneoplastic T-cell populations, we invariably found similar light scatter patterns among the various T-cell subsets. This feature is particularly helpful when the immunophenotypic aberrations on a tumor cell population are otherwise subtle; the presence of a distinctly different light scatter pattern on such a population is essentially diagnostic of T-cell neoplasia.

The distribution of CD4- and CD8-expressing cases in the present study was similar to that in previous studies. 8,10,11,14,15,32,33 Of 46 cases in which the neoplastic population was identified by flow cytometry, 65% expressed CD4, 24% expressed CD8, and 9% expressed neither. Only a single case (2%) showed dual expression of CD4 and CD8. This was also an uncommon phenomenon in most previous studies. 8,10,11,14,32,33 The major exception being the report of Hastrup et al, 15 in which 13 (17%) of 76 cases coexpressed CD4 and CD8. The explanation for this discrepancy is unclear, but it may have resulted from difficulty discriminating admixtures of CD4 and CD8 cells from coexpression on the same cell population.

Only 4 of 50 PTCNs in the present study lacked immunophenotypic aberrancy. A detailed analysis of these cases suggested that 2 of the 4 resulted from sampling error. In 1 case, diagnosed as an angioimmunoblastic T-cell lymphoma, the negative gene rearrangement analysis and benign clinical course suggest the possibility that this was a reactive proliferation. However, since the consensus diagnosis among several hematopathologists was lymphoma, the case was retained in the present series. Finally, a single case of unequivocal lymph node involvement by MF lacked immunophenotypic aberrancy, and a later analysis of overtly involved peripheral blood yielded the same result. Our results would suggest, therefore, that the vast majority of PTCNs are immunophenotypically aberrant by flow cytometric analysis but that cases exist that lack detectable aberrancy by the method we have outlined.

There have been several studies that used an approach similar to ours in the characterization of PTCN, all focusing on MF/Sézary syndrome. 35-37 Kuchnio et al 35 reported 2 discrete T-cell populations based on the intensity of CD3 expression in 4 of 5 patients with Sézary syndrome. They interpreted the bright CD3 population to represent the neoplastic clone; 3 of these populations lacked CD7, and the fourth lacked CD2. Bogen et al 36 reported discrete populations with decreased CD3 and/or CD4 expression in 7 of 10 patients with Sézary syndrome. The size of this population generally correlated well with the number of Sézary cells determined by nuclear contour analysis. The source of the discrepancy between the results of Kuchnio et al 35 vs those of Bogen et al 36 (increased vs decreased CD3 intensity) is not clear, although the result in the latter study is corroborated by a recent study by Edelman and Meyerson, 37 in which populations with diminished CD3 expression were detected in 19 of 44 specimens from patients with MF/Sézary syndrome. These authors reported 100% concordance between the detection of aberrant CD3 populations and the morphologic detection of neoplastic cells. While we did not attempt to correlate immunophenotypic findings with specific subtypes of PTCN in the present study because of few cases in any one REAL classification category, a survey of the 6 MF cases in our series revealed dim CD3 in 2 cases and dim CD4 in another 2; none had bright CD3. In contrast with the qualitative approach used by us and the studies described, Ginaldi et al 38 used a microbead method to quantify CD3 in several types of T-cell leukemias. Oddly, they detected decreased density of CD3 expression in all forms of T-cell leukemias except Sézary syndrome.

Given the sensitivity of multicolor flow cytometry in detecting aberrations in PTCNs, it represents a potentially useful adjunct in the diagnosis of PTCN. Certainly there are many examples of PTCN in which the infiltrate is overtly malignant on a morphologic basis, and all that remains in such cases is to characterize the lineage of the neoplastic cells. However, a subset of tumors show substantial histologic overlap with other reactive and neoplastic proliferations, and it is in these scenarios that flow cytometry might allow rapid confirmation of the neoplastic nature of a proliferation. We have encountered several examples of unsuspected T-cell neoplasia (misinterpreted as reactive or as Hodgkin lymphoma) in which the referring pathologist was first alerted to the possibility of T-cell lymphoma based on the immunophenotypic analysis. Another area in which flow cytometric immunophenotyping could potentially make an impact is in the interpretation of fine-needle aspiration specimens. Recent publications have addressed the usefulness of flow cytometry in the diagnosis and classification of lymphoid neoplasms in fine-needle aspiration specimens, but these have largely focused on B-cell neoplasms. 39-45 While no fine-needle aspiration specimens were included in the present series, the results that we have presented should be applicable to this technique.
For the method we have outlined to be clinically useful, in addition to being sensitive it must be highly specific. The T-cell immune response is highly complex, and it contains numerous small subsets based on expression of commonly assayed T-cell antigens. While these are generally small, they may be expanded in certain tissue sites, as well as in response to pathologic stimuli. These populations, such as gamma/delta T cells, CD4/CD8 dual-negative alpha/beta T cells, CD4/CD8 dual-positive T cells, CD2− T cells, CD5− T cells, and CD7− T cells, have been characterized by other investigators.17 To ensure that we were not inadvertently identifying expansions of normal subsets as neoplastic populations, we believed it was necessary to survey nonneoplastic T-cell populations in a variety of sites to provide ranges for our patient cohort. As with the neoplastic T-cell populations, these subsets were characterized and enumerated based on the formation of distinct clusters in multiparameter analysis. It should be noted that these are not normal ranges, in that they were obtained from a series of patient specimens submitted for a variety of pathologic processes; these ranges would be expected to differ based on the particular patient mix studied. We have chosen to include specimens involved by non–T-cell neoplasia, as well as specimens from patients with immune derangements, in order to encompass the range of findings seen in nonneoplastic T cell populations. Our goal was not to distinguish normal from abnormal, but rather to distinguish neoplastic from nonneoplastic. Of note in this regard, most outliers for each subset occurred in association with non–T-cell neoplasia. To be conservative, we considered T-cell populations to be aberrant only when they were expanded beyond the highest value we observed in nonneoplastic samples. Notably, only 3 PTCNs were considered aberrant solely on the basis of expansion of otherwise immunophenotypically normal populations beyond our established site-specific limits. The remainder showed aberrancy patterns not observed in nonneoplastic T-cell populations.

While a detailed discussion of nonneoplastic T-cell subpopulations is beyond the scope of the present article, a few points are worth noting, as they are particularly relevant to the interpretation of patient specimens. Deletion of CD7 has commonly been used as evidence of T-cell neoplasia. However, a large proportion of CD4+ cells may lack CD7 in blood, marrow, and lymph nodes (Table 3). Also, while we did not assess any skin specimens in our survey, the majority of normal dermal and epidermal T cells have been reported to lack CD7.46,47 Thus, expansions of CD7−/CD4+ cells should be interpreted with extreme caution when other immunophenotypic aberrations are not present.

Another subset that may be expanded to surprisingly large size is that of CD4 and CD8 dual-negative cells. Dual-negative T cells include alpha/beta and gamma/delta components; previous studies have shown that gamma/delta T cells represent 88% to 98% of the dual-negative population.28 Gamma/delta T cells may be expanded in a variety of infectious, autoimmune, immunodeficient, and neoplastic states.23,48-51 Notably, they may be expanded in early HIV infection. Given that dual negativity for CD4 and CD8 has been used in the past as a criterion for malignancy, awareness that they may be markedly expanded is important to avoid misdiagnosis. Interpretation is further complicated by the fact that gamma/delta T cells show variability in their expression of CD5. The majority of gamma/delta T cells in most patients express CD5, although at a lower level than alpha/beta T cells.52,54 In our experience, the gamma/delta T cell cluster shows distinctly dimmer CD5, but with substantial overlap with the alpha/beta cluster. In addition to the dim CD5 population, there is a discrete minority population of gamma/delta T cells that lack CD5.52,54

T cells have been shown to down-regulate CD3, CD4, and CD8 on activation.54-57 Underexpression of these antigens also has been documented in HIV-infected patients.55 This is only of importance for diagnostic purposes if these variations produce discrete subpopulations, which we have generally not found to be the case in clinical practice. During our survey of nonneoplastic T cells, we documented discrete populations with abnormal CD3 intensity in approximately 6% of blood and bone marrow specimens, but not in lymph node, spleen, or bowel specimens. However, only 1 of 14 was abnormally dim. The remaining 13 cases with abnormal CD3 intensity had abnormally bright expression. Interestingly, all 11 that were assessed for CD2 were also CD2−, and the 4 cases for which we could specifically assess CD4 and CD8 were either dual-negative or dim CD8+. Cases with these populations were significantly overrepresented among patients with B-cell neoplasia. We suspect these represented gamma/delta T cells, although we have not directly tested this hypothesis. Gamma/delta T cells have been reported to be expanded in association with non–T-cell lymphoid neoplasia.49 In addition a proportion of gamma/delta T cells may lack CD2 in some circumstances.17 In only 1 case did CD3(bright) cells represent more than 10% of T cells, and this was in the peripheral blood of a patient with AML in which they represented 57.6% of T cells; the T cells in turn only accounted for 2.8% of peripheral blood leukocytes in this case.

We have documented the usefulness of MPFC in the assessment of T-cell neoplasia. We found this to be a highly sensitive technique, demonstrating aberrancy in the large majority of cases across the spectrum of T-cell neoplasia. In addition, when interpreted in the context of detailed knowledge of normal and reactive T-cell populations it is highly specific.

From the University of Texas Southwestern Medical Center, Dallas.
References


