Flow Cytometric Immunophenotypic Assessment of T-Cell Clonality by $V_\beta$ Repertoire Analysis in Fine-Needle Aspirates and Cerebrospinal Fluid

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Abstract

Flow cytometric T-cell receptor $V_\beta$ repertoire analysis (TCR-$V_\beta$-R) is a sensitive method to detect T-cell clonality; however, its implementation in low-cellularity specimens has not been established. We developed a strategy to use TCR-$V_\beta$-R in cerebrospinal fluid (CSF) and fine-needle aspirate (FNA) specimens. Initially, full TCR-$V_\beta$-R was evaluated in diagnostic/screening specimens from 8 patients with T-cell neoplasia to determine tumor-specific TCR-$V_\beta$ protein expression. Subsequently, an abbreviated, patient-specific TCR-$V_\beta$-R evaluation was performed in 17 paucicellular specimens from the patients (8 CSF, 9 FNA) for staging and monitoring of minimal residual disease (MRD). A single cocktail containing 3 anti-$V_\beta$ antibodies (1 tumor-specific and 2 negative controls) in combination with other antibodies chosen to help gate on atypical T cells is highly sensitive and specific for detecting low-level neoplastic T-cell involvement in paucicellular specimens. This TCR-$V_\beta$-R strategy is valuable in staging and evaluating MRD in patients with T-cell non-Hodgkin lymphoma.

The diagnosis of lymphoma is frequently based on evaluation of small biopsy specimens, fine-needle aspirates (FNAs), and body fluids (ie, cerebrospinal fluid [CSF], vitreous humor, serous cavity effusions) in which involvement by hematopoietic malignancies may be difficult to document by morphologic examination alone. Flow cytometric immunophenotyping (FCM) improves the sensitivity of detection of non-Hodgkin lymphoma in CSF and FNAs.¹⁻⁶ Furthermore, because the World Health Organization classification incorporates immunophenotypic criteria, FCM evaluation of FNAs assists in detection and diagnostic subclassification of lymphoma.¹,²,⁷ FCM is even an independent prognostic indicator in patients at risk of having central nervous system involvement by aggressive B-cell lymphoma⁴ in that patients with positive CSF by FCM have a higher rate of central nervous system relapse and death than do patients with FCM– disease.

The small number of cells in FNA and CSF specimens necessitates abbreviated panels. This is easily accomplished in B-cell lymphoproliferative disorders by demonstration of restricted surface immunoglobulin light chain expression in a B-cell population. Mature T-cell lymphomas and leukemias, however, are more difficult to diagnose based on the need to examine a greater number of immunophenotypic markers for diagnosis; thus, the application of FCM to low-cellularity specimens has not been well established in these diseases. FCM evaluation of mature T-cell neoplasms is mainly based on aberrant expression levels of pan–T-cell antigens (eg, CD2, CD3, CD5, and CD7) or of T-cell subset restriction (eg, CD4, CD8, CD26), necessitating a comprehensive panel of antibodies.⁸,⁹ This may not be possible in paucicellular samples like CSF and FNA, even using multicolor FCM.
T-cell clonality can be detected through immunophenotypic analysis of the T-cell receptor Vβ repertoire (TCR-Vβ-R).10-14 All T cells in a clonal αβ T-cell population possess the same VDJ segment and, therefore, have identical, or “monoclonal,” Vβ protein expression. Since commercial fluorescently labeled antibodies are available against the majority of the human class-specific V segments for the TCR-β chain (Vβ), it is possible to use FCM to detect expanded T-cell populations that show a pathologic restriction in Vβ usage. Abnormal T-cell populations can be studied by a panel of anti-Vβ antibodies to determine clonality in the immunophenotypically defined abnormal T cells. This is called Vβ repertoire analysis.

The distribution of Vβ classes in normal CD4+ or CD8+ T cells is well defined.15,16 Expansion of a Vβ population in CD4+ or CD8+ T cells is consistent with a clonal T-cell population, similar to an expansion of κ- or λ-restricted B-cells in a monoclonal B-cell population. Furthermore, a large population of αβ T cells expressing surface CD3 but failing to express any of the Vβ proteins detectable by current TCR-Vβ-R assays (detect 70% of normal T cells) is abnormal and consistent with a clonal T-cell population expressing one of the Vβ proteins not studied (normally present in only 30% of T cells).11-14 Vβ repertoire analysis has also been demonstrated to be highly sensitive in the detection of minimal residual disease.10 Thus, Vβ repertoire analysis by FCM is an exciting new tool in the diagnosis of T-cell lymphoproliferative processes.

Previous studies of TCR-Vβ-R testing for the diagnosis and staging of T-cell neoplasms have focused on cellular specimens such as peripheral blood, bone marrow, and lymph node biopsy specimens. The usefulness of TCR-Vβ-R testing in paucicellular specimens such as CSF and soft tissue FNAs has not been examined. In the current study, TCR-Vβ-R testing using an abbreviated panel of antibodies was applied to CSF and FNA specimens from a series of patients with T-cell leukemia/lymphoma in which the specific Vβ class of the neoplastic T cells was known from initial diagnostic evaluation. FCM TCR-Vβ-R testing of paucicellular specimens provides increased ease of diagnosis and allows precise determination of the extent of involvement with mature T-cell leukemia/lymphoma.

### Materials and Methods

#### Specimens Evaluated

Initial comprehensive screening FCM was performed on peripheral blood specimens from 8 patients with a confirmed diagnosis of a mature T-cell malignancy to determine a tumor-specific immunophenotype for each person and the specific TCR-Vβ protein expression. The cases included 6 patients with adult T-cell leukemia/lymphoma associated with HTLV-1 infection (ATLL) and 2 patients with peripheral T-cell lymphoma, not otherwise specified. Classification of the T-cell malignancy was based on the World Health Organization criteria for hematologic malignancies using a combination of morphologic, immunohistochemical, molecular, and FCM studies.

The presence of clonal T cells in the screening peripheral blood sample was confirmed by FCM TCR-Vβ-R testing and concurrent molecular studies for TCR-γ gene rearrangement by a previously described polymerase chain reaction assay.17 Limited Vβ repertoire analysis for T-cell clonality was performed on 17 subsequent low-cellularity specimens (8 CSF; 9 FNA [7 skin nodules, 1 lymph node, and 1 soft tissue mass]) from the 8 patients for the purposes of staging or quantitation of response to therapy.

All patients signed institutional review board–approved informed consent for the testing performed. Clinical data were obtained through medical record review and by contacting the patient’s National Institutes of Health staff physicians.

### Immunophenotyping

CSF specimens (1-4 mL) were immediately placed in 4 mL of RPMI with 10% fetal calf serum and transported to the laboratory at room temperature. CSF specimens were pelleted and stained within 2 hours of collection with antibody panels based on screening immunophenotype and cellularity of the specimen. There was no gross evidence of blood contamination of the CSF specimens, and erythrocyte lysis was not performed. FNA specimens were immediately placed in RPMI and transported to the laboratory at room temperature. FNA specimens were pelleted and stained within 12 hours of collection with antibody panels based on screening immunophenotype and cellularity of the specimen. When erythrocytes were present in FNAs, they were lysed by incubating with lysing solution (150 mmol/L ammonium chloride, 10 mmol/L potassium bicarbonate, 0.1 mmol/L EDTA) for 10 minutes at room temperature (maintained at 21°C-23°C) at a ratio of 1:9 (volume of sample/volume of lysing solution). Peripheral blood specimens were lysed (as for FNAs) and stained within 12 hours of collection. Specimens were stained for 30 minutes at room temperature (maintained at 21°C-23°C) with a cocktail of 4 antibodies (antibody concentration according to manufacturer’s recommendations) as previously described and according to Clinical Laboratory Standards Institute document H43-A recommendations.18,19

The initial screening peripheral blood panels included antibodies against CD2, CD3, CD4, CD5, CD7, CD8, CD10, CD14, CD16, CD19, CD20, CD25, CD26, CD27, CD30, CD45, CD52, CD56, CD57, γδ TCR, αβ TCR, κ, and λ. In addition, screening panels included the entire IOTest Beta Mark TCR-Vβ Repertoire kit (Beckman Coulter, Miami,

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The CSF and FNA antibody panels were chosen based on the number of cells, the diagnosis, and previous immunophenotypic data. Clone-specific \( V_\beta \) antibodies in a cocktail with 2 additional \( V_\beta \) negative controls (antibodies against 2 \( V_\beta \) proteins not expressed by the neoplastic T cells) were used in the CSF and FNA panels. All TCR-\( V_\beta \) antibodies were mixed in cocktails with anti-CD3 peridinin chlorophyll-a protein (PerCP) and anti-CD4 and/or anti-CD8 allophycocyanin (APC). In occasional cases, anti-CD5 or anti-CD7 APC antibodies were substituted for anti-CD4 or anti-CD8 where previous FCM indicated they were more valuable in isolating the abnormal T-cell population.

All cells were fixed in 1.0% paraformaldehyde and stored at 4°C for up to 12 hours before acquisition. Four-color cytometry was performed using a BD Biosciences (San Jose, CA) FACSCalibur flow cytometer (sensitivity of fluorescent detectors monitored using standard beads according to the manufacturer’s recommendations). Data (collected in list mode) were analyzed with FCS Express (De Novo Software, Los Angeles, CA). Relevant cell populations were analyzed by examining forward angle light scatter, side angle light scatter, CD3, CD4, CD8, and other characteristic markers for each specific disease entity (eg, CD4+/CD7–/CD25-bright for ATLL). Normal lymphoid cells in the specimens served as internal positive and negative controls (eg, normal B cells served as negative controls for T cell–directed antibodies) and as a standard for normal antibody binding intensity.

Diagnostic abnormal T-cell immunophenotypic features were identified in each individual case and used to develop specific tumor gating strategies for analysis of TCR-\( V_\beta \) antigen usage.8,10 These features included aberrant dim or bright expression of antigens (eg, dim CD3 or bright CD4) or lack of expression of appropriate antigens (CD4– and CD8–, CD2–, CD5–, or CD7– CD3+ cells; CD26–CD4+ and CD3+ cells). Based on correlation with previously published studies, clonality in this assay was defined as the expression of a single TCR-\( V_\beta \) in greater than 50% of the T cells in any analyzed population (gated T cells).10–14 The results of immunophenotypic and TCR-\( V_\beta \)-R analysis were correlated with morphologic evaluation.
Cytopathologic Analysis

Fresh CSF and FNA specimens were refrigerated and processed within several hours of collection. Cytocentrifuged slides were prepared by centrifugation of undiluted specimen at 500 rpm for 5 minutes. The slides were then air dried and stained with Diff-Quik (Dade, Aguada, PR). Samples were concentrated or diluted in RPMI 1640 when required. FNA samples were diluted in RPMI 1640 if needed. Each case was evaluated independently by the same cytopathologist (A.C.F.) and classified as positive for malignancy, negative for malignancy, or indeterminate (atypical or “suspicious” morphologic features).

Results

Initial Flow Cytometric Screening of Peripheral Blood

Atypical cells were identified by using a multiparametric approach on the basis of dim CD3, bright CD4, coexpression of CD4 and CD8, CD7 negativity, increased CD25 expression, and CD26 negativity in CD4+ T cells. Absence of CD26 was particularly useful in cases in which abnormal levels of CD3 expression were not distinctly apparent. A complete TCR-Vβ-R analysis was performed at initial screening using a gating strategy based on the identified specific abnormal immunophenotypic features of the neoplastic cells in each case. The initial immunophenotypic data, including the Vβ clone expressed by the neoplastic T cells, is shown in Table 1.

Flow Cytometric Evaluation of CSF and FNA Samples

Of 8 patients included in this study, 3 had 2 or more evaluations (2-5) at varying intervals. Neoplastic T cells were detected in all FNA specimens and in 6 of 8 CSF specimens (CSF samples from cases 6 and 8 were negative). The percentage of lymphoid cells that were neoplastic ranged from 1.75% to 85% in FNA specimens and from 56% to 100% in CSF specimens. In all 15 positive samples (6 CSF and 9 FNA), T-cell clonality was detected by demonstration of expression of a single Vβ protein by greater than 50% of the gated T cells (CSF specimens, mean, 85.46% and range, 72.37%-93.26% of gated T cells; FNA specimens, mean, 79.27% and range, 53.4%-100% of gated T cells). The immunophenotypic profile of the neoplastic T cells, including TCR-Vβ-R analysis, is shown in Table 2 for all positive specimens.

Correlation of Flow Cytometric Results with Cytomorphologic Findings

Cytomorphologic evaluation was available for all CSF specimens and for 8 of 9 FNA specimens studied. Cytomorphologic results were atypical in both CSF specimens FCM− for T-cell neoplasia. Of the 6 CSF specimens FCM+ for T-cell neoplasia, 1 was positive by cytomorphologic evaluation, 3 were negative by cytomorphologic evaluation, and 2 were suspicious or atypical by cytomorphologic evaluation. All 9 FNA specimens were FCM+. In the 8 FNA specimens with concurrent cytomorphologic results, 4 were positive by cytomorphologic evaluation and four were suspicious or atypical.

Discussion

Multiparametric FCM can be highly sensitive for the detection of T-cell leukemia and lymphoma based on immunophenotypic aberrancies such as absent, diminished, or abnormally increased expression of T-cell antigens or presence of aberrant antigens. However, in low-cellularity specimens like CSF or other body fluids, it is not possible to perform

<table>
<thead>
<tr>
<th>Table 1</th>
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<th></th>
<th></th>
<th>Gated T-Cells, Vβ (%)</th>
</tr>
</thead>
</table>
| Diagnosis/Case No. | Specimen Type | CD2 | CD3 | CD4 | CD5 | CD7 | CD8 | CD25 | CD26 | CD57 | Vβ
| Adult T-cell leukemia/lymphoma | PB | + | ± | + | + | – | – | ++ | – | – | 1 | 72.5 |
| 1 | PB | + | ++ | ++ | + | + | + | – | – | + | 17 | 70.1 |
| 2 | PB | + | ± | + | + | – | – | ++ | – | – | 13.1 | 82.7 |
| 3 | PB | + | + | + | + | – | – | ++ | – | – | 17 | 52.4 |
| 4 | PB | + | + | + | + | – | – | ++ | – | – | 17 | 99.6 |
| 5 | PB | + | ± | + | + | + | ++ | – | – | – | 5.2 | 51.7 |
| 6 | PB | ± | ± | + | ++ | – | – | ++ | – | – | 4 | 83.7 |
| Peripheral T-cell lymphoma, not otherwise specified | PB | + | + | + | + | + | + | – | – | + | 17 | 85.3 |

PB, peripheral blood; −, negative; ±, dim; +, positive; ++, bright positive.
* Partial.
Tembhare et al / Vβ Analysis in Cytology Specimens

Table 2
Immunophenotypic Profile of Neoplastic T Cells in FNA and CSF Specimens

<table>
<thead>
<tr>
<th>Specimen/Case No.</th>
<th>CD2</th>
<th>CD3</th>
<th>CD4</th>
<th>CD5</th>
<th>CD7</th>
<th>CD8</th>
<th>CD25</th>
<th>CD45</th>
<th>CD57</th>
<th>Vβ</th>
<th>Gated T Cells, Vβ+ (%)</th>
<th>Tumor Cells (%)</th>
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<tbody>
<tr>
<td>FNA</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>1</td>
<td>+</td>
<td>±</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>−</td>
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<td>ND</td>
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<td>ND</td>
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<td>±</td>
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<td>ND</td>
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<td>ND</td>
<td>ND</td>
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<td>+</td>
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<td>+</td>
<td>−</td>
<td>ND</td>
<td>+</td>
<td>−</td>
<td>17</td>
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<td>ND</td>
<td>+</td>
<td>+</td>
<td>ND</td>
<td>+</td>
<td>−</td>
<td>ND</td>
<td>+</td>
<td>−</td>
<td>17</td>
<td>93.3</td>
<td>83</td>
</tr>
</tbody>
</table>

CSF, cerebrospinal fluid; FNA, fine-needle aspirate; ND, not done; −, negative; ±, dim; +, positive; ++, bright positive.

Table 3
Flow Cytometric and Cytomorphologic Detection of T-Cell Neoplasia

<table>
<thead>
<tr>
<th>Cytomorphology</th>
<th>Total No. of Cases</th>
<th>Flow Cytometry</th>
<th>Positive</th>
<th>Indeterminate*</th>
<th>Negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>FNA specimens</td>
<td></td>
<td>Positive</td>
<td>4</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>Negative Total No. of cases</td>
<td>4</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>8</td>
</tr>
<tr>
<td>CSF specimens</td>
<td></td>
<td>Positive</td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Negative Total No. of cases</td>
<td>1</td>
<td>2</td>
<td>0</td>
<td>2</td>
<td>8</td>
</tr>
</tbody>
</table>

* Indeterminate, “suspicious” or atypical morphologic features.

Improving the sensitivity of techniques for diagnostic evaluation of FNA and CSF specimens is therefore highly desirable.

Flow cytometric TCR-Vβ-R analysis is a rapid quantitative method to detect clonality of T-cell populations in patients with suspected T-cell lymphoproliferative disorders, providing a high degree of sensitivity and specificity for T-cell clonality assessment. Typically, TCR-Vβ-R analysis uses an extensive panel of Vβ antibodies in combination with other antibodies useful to identify malignant T cells. However, once the specific Vβ protein expressed by the neoplastic T cells is known, they can be detected in any low-cellularity specimen via a single Vβ-specific cocktail (ie, a cocktail that includes the neoplastic T cell–specific Vβ antibody and 2 other negative control Vβ antibodies) (Image 1 and Table 2).

In this study, we determined the usefulness of FCM single-cocktail TCR-Vβ-R analysis in the diagnostic evaluation of 17 paucicellular specimens such as CSF and FNA from 8 patients with T-cell non-Hodgkin lymphoma. We determined the immunophenotype and specific Vβ protein expressed by the neoplastic T cells at the time of initial screening using a comprehensive panel of antibodies and complete TCR-Vβ-R analysis. An expansion of a single Vβ family to an abnormally high percentage of the CD4+/CD3+ or CD8+/CD3+ T cells is considered evidence of T-cell clonality. We have previously demonstrated that expression of a single Vβ protein in greater than 50% of a specific immunophenotypically abnormal gated T-cell population is evidence of T-cell clonality.

In the present study, analysis gates were drawn around T cells with an atypical immunophenotype matching that observed in the original screening specimen (as previously
described\(^1\)), and the same 50% \(V_\beta\) expression cutoff was applied to assessment of the \(V_\beta\) protein pattern. In all specimens analyzed, greater than 50% of the atypical T cells were restricted to a single \(V_\beta\) protein (Table 2). These results showed that a single cocktail containing 3 anti-\(V_\beta\) antibodies, 1 tumor-specific and 2 negative controls, in combination with other antibodies to delineate the atypical T cells is highly sensitive for the evaluation of low-level T-cell non-Hodgkin lymphoma involvement in paucicellular specimens.

Flow cytometric TCR-\(V_\beta\)-R analysis was more sensitive than cytomorphologic evaluation alone for detecting T-cell non-Hodgkin lymphoma in paucicellular specimens (Table 3). FCM detected clonal T-cell populations in all 8 FNA specimens in which concurrent cytomorphologic results were available, while cytomorphologic results were positive in only 4 cases (50%) and indeterminate in the remainder. This is likely due, in part, to the fact that the percentage of neoplastic cells identified was quite low in select FNA samples. The percentages ranged from 1.75% to 85% (mean ± SD, 25.7% ± 28.8%).

FCM detected clonal T-cell populations in 6 of 8 CSF specimens studied. Among these 6 FCM+ cases, cytomorphologic results were positive in only 1 (17%), indeterminate in 2 (33%), and negative in 3 (50%), even though there was generally a higher percentage of neoplastic cells in CSF compared with FNA specimens. The mean ± SD percentage of neoplastic cells in CSF samples was 83.1% ± 14.7% (range, 56%-100%). Of the 2 FCM– CSF specimens, cytomorphologic results were indeterminate.

The greater sensitivity of FCM detection of T-cell non-Hodgkin lymphoma in CSF is not surprising. There is extensive literature definitively demonstrating that FCM is more sensitive than cytomorphologic evaluation for detecting CSF involvement with B-cell non-Hodgkin lymphoma.\(^4,5,21,24\)

Studies comparing FCM and cytomorphologic evaluation in CSF from patients with T-cell non-Hodgkin lymphoma are less common. In a study by Schinstine et al.,\(^22\) only 2 of 6 CSF specimens diagnosed as positive for malignant T cells by FCM were found to be positive by morphologic evaluation.

There is little literature available on FCM evaluation of FNAs for T-cell non-Hodgkin lymphoma. FCM evaluation using a standard 3-color antibody panel approach demonstrated aneuploidy and an abnormal immunophenotype in a single FNA demonstrated by cytomorphologic evaluation to be involved with a T-cell non-Hodgkin lymphoma. In another report, concurrent FCM and cytomorphologic data were available for 8 FNAs from patients with ATLL.\(^23\) While a comparison of sensitivity cannot be made because cases were selected based on positive cytomorphologic results, FCM was positive for neoplastic T cells in all 8 cases. Although the present study is limited to only 17 samples, with the majority procured from patients with ATLL (an uncommon T-cell neoplasm), it expands on the existing literature, providing solid evidence that FCM TCR-\(V_\beta\)-R analysis is more sensitive than morphologic evaluation in evaluating paucicellular FNAs and CSF for T-cell non-Hodgkin lymphoma.

FCM TCR-\(V_\beta\)-R analysis is a sensitive and specific method to detect clonal T-cell non-Hodgkin lymphoma. Initial determination of the specific TCR-\(V_\beta\) expressed in a neoplastic T-cell population analysis requires an extensive panel of \(V_\beta\) antibodies in a cocktail targeting antigens useful in identifying malignant T cells. However, once the specific \(V_\beta\) protein expressed by the neoplastic T cells is known, it is possible to detect the presence of tumor cells in any low-cellularity specimen (eg, CSF and FNA) via a single \(V_\beta\)-specific cocktail that includes the neoplastic T cell–specific \(V_\beta\) antibody and 2 other negative control \(V_\beta\) antibodies. FCM TCR-\(V_\beta\)-R analysis is more sensitive than cytomorphologic evaluation alone in detecting T-cell non-Hodgkin lymphoma in these paucicellular specimens; therefore, it is highly valuable in the staging and evaluation of minimal residual disease in patients with T-cell non-Hodgkin lymphoma.

References

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