

A Comparison of Morphologic Features, Flow Cytometry, TCR- V_{β} Analysis, and TCR-PCR in Qualitative and Quantitative Assessment of Peripheral Blood Involvement by Sézary Syndrome

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Abstract

The strengths and weaknesses of various laboratory methods for peripheral blood (PB) Sézary cell quantitation have not been compared rigorously. In this study, manual Sézary cell counting, qualitative and quantitative flow cytometry, T-cell receptor (TCR) V_{β} flow cytometry, and TCR polymerase chain reaction were performed on PB specimens from 11 patients with Sézary syndrome (SS), 9 with reactive erythroderma, 6 with mycosis fungoides, and 11 healthy control subjects. These methods identified neoplastic cells in more than 90% of SS cases. The diagnostic specificities of these tests varied; they were enhanced by applying criteria proposed by the International Society for Cutaneous Lymphoma. Comparison of sequentially analyzed specimens from 6 patients with SS revealed that although the absolute number of clonal cells was reduced, in some cases, these cells still constituted the vast majority of the CD4+ T-cell subset, suggesting that quantitative subset analysis might be sufficient to monitor changes in the PB tumor burden.

Peripheral blood (PB) involvement by erythrodermic cutaneous T-cell lymphoma (E-CTCL) was first recognized by Sézary in 1938 with the description of circulating large, atypical cells (“cellules monstreuses”) in a patient with diffuse erythroderma.¹ The term *Sézary syndrome* (SS) subsequently was coined to describe E-CTCL in leukemic phase usually associated with distinctive clinical features such as ectropion, palmar-plantar keratoderma, and lymphadenopathy. Since these early descriptions, it has been recognized that the degree of PB involvement by E-CTCL is an important prognostic variable, yet there is no universally accepted method to assess and quantify the number of circulating neoplastic T cells.^{2,3} Before the advent of ancillary immunophenotyping and molecular genetic techniques, Sézary cell counting represented the mainstay of disease detection and quantification. This approach is compromised by difficulties in recognizing Sézary cells, particularly those with small nuclei, and the fact that circulating small Sézary cells may be seen in normal PB and in reactive conditions.⁴⁻⁷

A number of studies have documented the usefulness of flow cytometric immunophenotyping (FCIP) in the detection of circulating Sézary cells. The most commonly described aberrancy in SS is loss of CD7, and others include the diminished or absent expression of CD2, CD3, and/or CD26.⁸⁻¹² Although these attributes are useful for identifying Sézary cells, they are present variably, and some, including down-regulated expression of CD7, have been found in reactive T-cell populations.¹³ For these reasons, the usefulness of qualitative immunophenotyping analysis to accurately quantify the number of circulating abnormal cells in SS remains unclear.

Establishing T-cell clonality remains a fundamental element in confirming a diagnosis of PB involvement by E-CTCL.

T-cell clonality traditionally has been assessed through polymerase chain reaction (PCR) and/or Southern blot analysis of T-cell receptor (*TCR*) gene rearrangements.¹⁴ Neither of these methods can reliably be considered quantitative and, unless preanalytic cell sorting methods are used, one cannot determine whether the clonal gene rearrangement detected resides within the cell population of interest.

FCIP using antibody panels directed against the TCR β chain variable region families (TCR-V β flow) can be used effectively to demonstrate clonality in PB specimens involved by a variety of T-cell neoplasms, including CTCL.¹⁵ An advantage of TCR-V β flow compared with molecular genetic techniques is that the reagents can be combined with antibodies to other T cell–associated antigens to allow for clonality assessment in specific T-cell populations. The use of TCR-V β antibodies to monitor changes in the PB tumor burden in patients with CTCL during treatment has been reported.^{16,17} As performed in most laboratories, however, the TCR-V β flow has preanalytic lysis and washing steps that make this test only semiquantitative.¹⁵ Flow cytometry can provide accurate quantitative results if special methods involving the use of calibration beads and unwashed cells are used.

The International Society for Cutaneous Lymphoma (ISCL) recently published recommended criteria for the diagnosis of PB involvement by E-CTCL/SS.¹⁸ In the present study, the PB specimens from 11 patients with SS were studied by morphologic Sézary cell assessment, FCIP, quantitative flow cytometric T-cell subset analysis, TCR-V β flow, and TCR PCR to determine their usefulness in the detection and quantification of involvement by E-CTCL/SS. The results were compared with those obtained from PB specimens from 11 healthy adult volunteers, 6 patients with mycosis fungoides, and 9 patients with benign dermatoses using proposed ISCL criteria to assess the diagnostic specificity of the results obtained. In addition, these studies were repeated in sequential specimens from 6 of 11 patients with SS to determine the optimal method to monitor the change in PB disease burden during treatment.

Materials and Methods

The study was approved by the Mayo Foundation Institutional Review Board, and all participants agreed to the use of their medical records. All studies were done prospectively; the 11 patients with SS all had diffuse erythroderma and a diagnostic skin biopsy including evidence of clonal *TCR* gene rearrangements. PB specimens from 11 healthy adult volunteers, 6 patients with biopsy-proven mycosis fungoides, and 9 patients with benign dermatoses and associated erythemic rashes were studied. The patients with mycosis fungoides all had the patch-plaque disease stage, and in 1 patient,

a primary cutaneous anaplastic large cell lymphoma, T-cell type, also had developed. The diagnoses in the benign dermatoses cases included nonspecific generalized dermatitis ($n = 3$), parapsoriasis ($n = 2$), and 1 case each of generalized eosinophilic dermatitis, psoriasis vulgaris, pityriasis rubra pilaris, and psoriasiform dermatitis.

Sézary Cell Counting

Sézary cell counts were performed in a blinded manner by hematology laboratory technical specialists using Wright-Giemsa–stained PB smears. These technical specialists are trained to identify as Sézary cells lymphocytes with hyperchromatic nuclei having folded and grooved nuclear membranes (“cerebriform” nuclei). These technologists routinely perform and report PB Sézary cell counts in the clinical laboratory when requested by a clinical care provider. For the clinical laboratory test and for the purposes of this study, Sézary cells as a percentage of the total lymphocytes was recorded. The results were reviewed by 1 hematopathologist (W.G.M.) to confirm the results.

Flow Cytometric Immunophenotyping

FCIP was performed according to previously described methods.¹⁹ T-cell phenotyping was performed using fluoro-chrome-conjugated antibodies to the following antigens: CD2, CD3, CD4, CD5, CD7, CD8, CD10, CD16, CD19, CD45, and κ and λ immunoglobulin light chains. Both 2-color flow cytometry using fluorescein isothiocyanate (FITC) and phycoerythrin (PE) antibody conjugates and 4-color flow cytometry using FITC, PE, allophycocyanin (APC), and peridinin chlorophyll-a protein (PerCP) were performed. All antibody conjugates were obtained from BD Biosciences, San Jose, CA.

The technique used for flow cytometric assessment of TCR-V β expression has been published.¹⁵ This method uses an 8-tube panel covering 24 known TCR-V β family specificities (3 TCR-V β antibodies per tube; IOTest Beta Mark TCR-V β Repertoire Kit, Beckman Coulter, Miami, FL). In addition to the TCR-V β antibodies, each tube also contained antibodies to CD3 (PerCP) and CD8 (APC) to allow for specific analysis of the TCR-V β repertoire in the CD8+ and CD8– T-cell subsets. Antibodies to CD4 were not used because this previously led to unacceptably high levels of nonspecific TCR-V β staining (discussed in detail previously¹⁵). The results were considered diagnostic of clonality if a single TCR-V β was expressed by more than 50% of a gated T-cell population or at a 10-fold or higher proportion than the normal maximum in a gated T-cell population. Cases in which greater than 70% of any gated T-cell population failed to react with any of the TCR-V β antibodies, presumably due to expression of a TCR-V β not recognized by the antibody panel, were considered consistent with clonality. Previous studies have demonstrated that

clonal *TCR* gene rearrangements can be detected in all cases with these patterns of *TCR-V β* reactivity.

The T-cell phenotyping and *TCR-V β* flow studies were performed on a FACSCalibur instrument (BD Biosciences), and the obtained data was analyzed using CellQuest software (BD Biosciences).

Quantitative T-Cell Subset Analysis

Quantitative flow cytometric T-cell subset analysis was performed by adding 50 μ L of anticoagulated whole blood to a tube containing a known, calibrated amount of internal standard fluorescent beads (TruCount tubes, BD Biosciences). The following antibody combinations were used in 3 separate tubes: tube 1, CD3 (FITC), CD8 (PE), CD45 (PerCP), and CD4 (APC); tube 2, CD3 (FITC), CD16 and CD56 (PE), CD45 (PerCP), and CD19 (APC); tube 3, CD3 (FITC), CD7 (PE), and CD45 (PerCP).

After incubation with antibody cocktails and addition of Multitest lysis reagent (BD Biosciences), the unwashed specimens were analyzed on a FACSCalibur instrument, and a minimum of 2,500 gated lymphoid events (CD45 and forward light scatter analysis) were collected. The data were processed using MultiSet software (BD Biosciences), which uses the internal calibration beads to calculate the percentage (of lymphoid cells) and total number of cells in each analyzed subset. The total lymphocyte count was determined by CD45 and side scatter gating, and the remaining subsets were quantified by antibody combinations as described in the “Results” section.

T-Cell Receptor PCR

PCR analysis was performed using 0.5 μ g of genomic DNA, Taq-Gold DNA polymerase (Applied Biosystems, Foster City, CA), and a mixture of 4 fluorochrome-labeled 5' primers specific for each of the *TCR γ* chain gene variable region families (*V γ 1*, *V γ 2*, *V γ 3*, and *V γ 4*) and two 3' primers specific for the *TCR γ* chain joining segments (*J1* and *J2*). We performed 40 PCR cycles on an MBS Satellite thermocycler

(ThermoHybaid, Ashford, England). The PCR products were analyzed on an ABI 3100 using GeneMapper software (Applied Biosystems). The *TCR* PCR was considered positive for a clonal rearrangement if there were 3 or fewer discrete peaks on the ABI tracing that were at least 4 times greater in amplitude than the polyclonal background banding pattern. The *TCR* PCR was considered equivocal if there were more than 3 such discrete peaks or if the discrete peak was greater than 2 times but less than 4 times the polyclonal background. Cases that did not satisfy these criteria were considered negative.

Results

The clinical and laboratory features of the various study groups are summarized in **Table 1**. The skin diseases associated with erythemic dermatoses in the reactive group are detailed in the “Materials and Methods” section. Among the 11 patients with SS, 4 were first studied at diagnosis. In the remaining 7 patients, the time from diagnosis to first study evaluation ranged from 2 to 96 months, with a median of 36 months. All 7 of these patients had received previous therapy; 6 of the 7 were treated with extracorporeal photopheresis (ECP), and the remaining patient received psoralen and long-wave UV radiation.

Sézary Cell Counting

Sézary cells were identified in all study groups (Table 1), including the majority of the healthy control cases; however, the percentage of Sézary cells and the absolute Sézary cell counts were considerably lower in the non-SS cases. Although a slightly higher percentage of Sézary cells was identified in the healthy control group compared with the other control groups, the differences in the percentage of Sézary cells among the control groups were not statistically significant when compared by using the Student *t* test (*P* > .05). In contrast, the differences in the percentage and calculated absolute

Table 1
Peripheral Blood Findings in the Study Groups*

	Healthy Control Subjects (n = 11)	Reactive Dermatoses (n = 9)	Sézary Syndrome (n = 11)	Mycosis Fungoides (n = 6)
Age (y)	39 (21-60)	69 (34-81)	73 (43-79)	50 (33-64)
Male/female ratio	5:6	4:5	8:3	2:4
Lymphocyte count ($\times 10^9$ /L)	1.6 (0.8-2.5)	1.7 (0.6-2.4)	3.7 (0.3-26.6)	1.4 (1.1-2.1)
Samples with Sézary cells identified	9 (82)	4 (44)	10 (91)	2 (33)
Sézary cells as percentage of lymphocytes	6 (0-14)	0 (0-10)	25 (28-75)	0 (0-8)
Samples with Sézary cells $\geq 20\%$	0 (0)	0 (0)	11 (100) [†]	0 (0)
Sézary cell count (cells/ μ L)	90 (0-210)	0 (0-80)	1,360 (175-14,100)	0 (0-160)
Samples with Sézary cells $\geq 1,000/\mu$ L	0 (0)	0 (0)	8 (73) [‡]	0 (0)

* Sézary counts were determined by manual differential of a peripheral blood smear performed by hematology technical specialists and reviewed by one of us (W.G.M.). Data are given as median (range) or number (percentage) unless otherwise indicated.
[†] Sézary syndrome vs reactive dermatoses, *P* < .001; Fisher exact test.
[‡] Sézary syndrome vs reactive dermatoses, *P* = .001; Fisher exact test.

number of Sézary cells between the SS cases and the control groups were statistically significant when compared by this method ($P < .0001$). The proposed ISCL criteria for PB involvement by SS included an absolute Sézary cell count of equal to or greater than 1,000/ μL .¹⁸ 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.^{20,21} These morphologic criteria for the diagnosis of SS were met by 8 (73%) and 11 (100%) of the SS cases respectively (Table 1). In contrast, none of the cases from the control groups satisfied either criterion.

The presence of large Sézary cells also has been described as a disease-specific finding in SS.²¹ Although large Sézary cells were not enumerated during Sézary cell counting, by qualitative assessment they were found in 5 of 11 Sézary cases and in none of the other groups. Of note, in 1 case, the presence of numerous large Sézary cells initially led to an erroneous undercounting because they were misinterpreted to be monocytes (Image 1). In this case, the Sézary cell count performed by the laboratory technologist was corrected after the PB smear was reviewed by a hematopathologist.

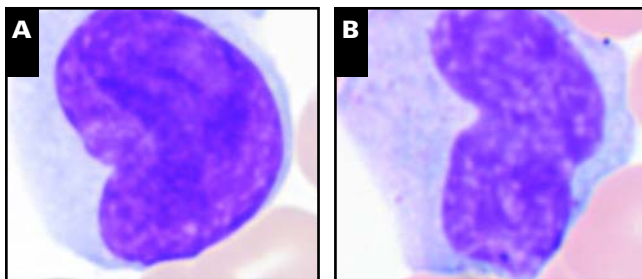


Image 1 (Case 4) Morphologic features of large Sézary cells. **A**, The Sézary cells were large with delicately reticulated chromatin (oil, $\times 1,000$). These attributes led the Sézary cells to be initially misinterpreted as monocytes on the manual differential count. **B**, A monocyte from the same smear is shown for comparison (oil, $\times 1,000$). Close examination of the cell shown in **A** revealed the narrow nuclear grooves pathognomonic of Sézary cells.

Table 2
Aberrant T-Cell Antigen Expression in 11 Cases of Sézary Syndrome*

Antigen	Complete Loss	Partial Loss	Diminished Intensity	Bright Intensity	Total Aberrant Cases
CD2	0 (0)	2 (18)	3 (27)	0 (0)	5 (45)
CD3	0 (0)	0 (0)	3 (27)	1 (9)	4 (36)
CD5	0 (0)	0 (0)	0 (0)	3 (27)	3 (27)
CD7	4 (36)	4 (36)	1 (9)	0 (0)	9 (82)

* Data are given as number (percentage). Complete loss indicates the antigen was not expressed by the entire cell population of interest; partial loss, the antigen was not expressed by a subset of the cell population of interest; diminished intensity, staining intensity for the antigen at least one-half log lower than normal T cells; and bright intensity, staining intensity for the antigen at least one-half log higher than normal T cells.

Flow Cytometric Immunophenotyping

Antigen expression by CD4+ T cells was assessed by 2- and 4-color FCIP in the SS cases and in all of the control groups. A CD4+ T-cell population with distinctively aberrant expression of T cell-associated antigens was present in 10 of 11 SS cases (Table 2). Aberrance in the expression of CD7 was most common, with partial or complete loss of this antigen in 8 of 11 cases (Image 2). In 2 cases, aberrance of CD7 expression was the only abnormality detected by FCIP. Selective gating on the phenotypically abnormal CD4+ T cells in 4-color flow cytometric studies demonstrated that in some cases, CD7 expression was down-regulated only in a subset of the neoplastic cells (Image 3). Other observed abnormalities in this group included (in order of frequency) diminished staining intensity or partial loss of CD2 (Image 2A, 5 cases), diminished or bright staining intensity for CD3 (Images 2B and 3, 4 cases), and abnormally bright expression of CD5 (Image 2B, 3 cases). In the 1 (case 4) (Table 3) and (Table 4) with immunophenotypically unremarkable CD4+ T cells, the patient had received previous ECP. The neoplastic cells in this case were distinguished by their abnormal forward and side light scatter characteristics (Image 4).

The only immunophenotypic abnormality detected in the CD4+ T cells among the control groups was partial loss of CD7 expression, which was present in 3 of the reactive dermatoses cases. The lack of diagnostic specificity of abnormalities of CD7 expression has been recognized previously.¹³ As a result, a threshold of 40% or greater of the CD4+ T cells exhibiting loss of CD7 has been suggested by the ISCL as a criterion for the diagnosis of SS.¹⁸ The percentage of CD7-/CD4+ T cells was higher in the SS cases than in the control groups. Of 11 SS cases, 5 exceeded the 40% cutoff; this criterion was not met in any of the reactive cases (Table 5).

Quantitative T-cell subset analysis also was performed using a combination of antibodies to CD3, CD4, and CD7 in an effort to more accurately enumerate the CD4+ T cells with loss of CD7. The percentages of CD4+/CD7- cells estimated by FCIP and T-cell subset analysis were similar (data not shown). Quantitative T-cell subset analysis was most effective when the antibody combinations allowed for a clear distinction of cell populations, such as with CD4+/CD8+ T cells. In contrast, the

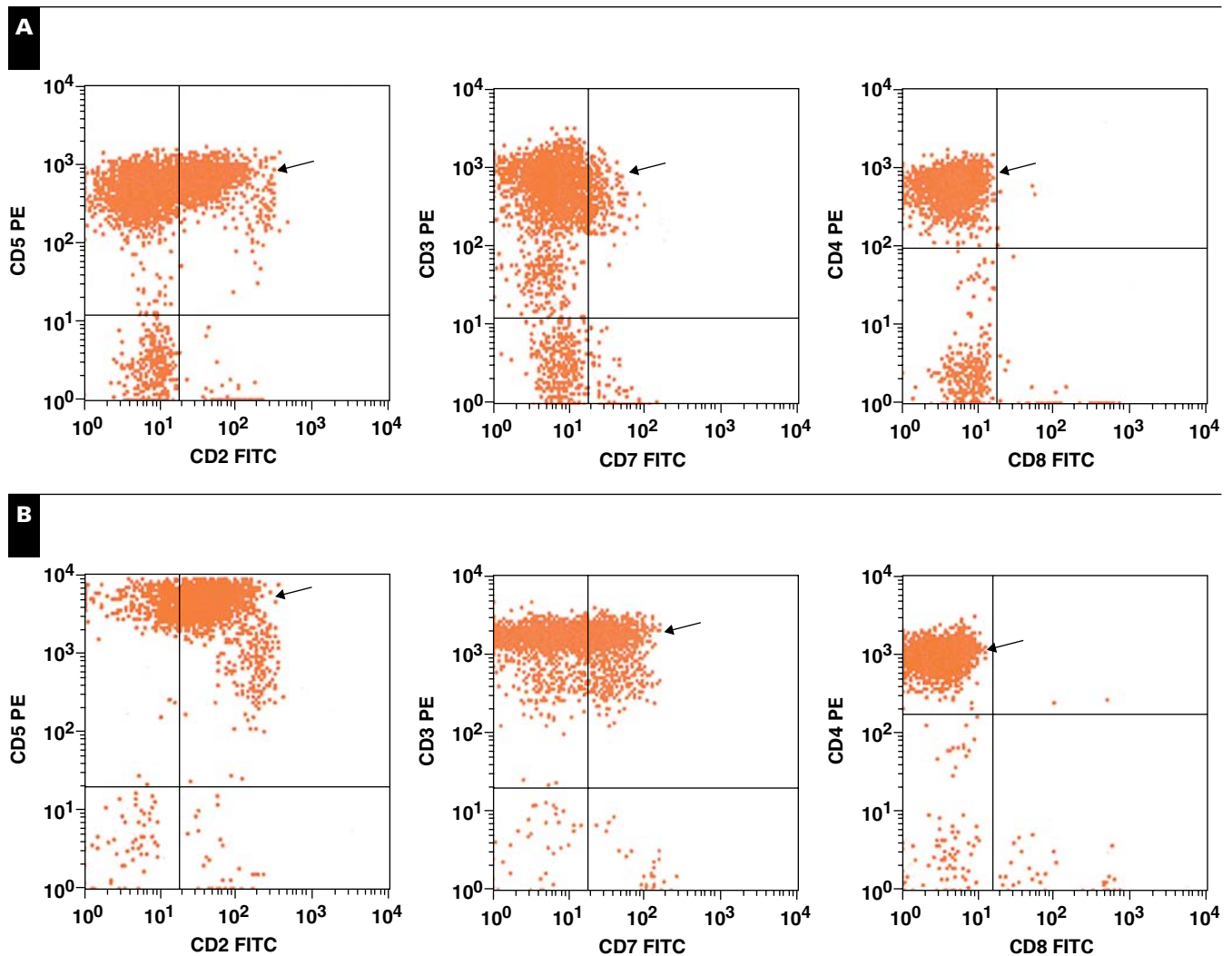


Image 2 Two-color flow cytometric immunophenotyping in Sézary syndrome. Flow cytometric evaluation of peripheral blood specimens from 2 Sézary syndrome cases revealed the presence of a CD4⁺ T-cell population with abnormally diminished expression of CD7 in both cases. **A**, The T cells also showed diminished expression of CD2; a minor subset of these also showed diminished staining with antibodies to CD3 (arrows). **B**, In contrast, the T cells showed abnormally bright staining with antibodies to CD3 and CD5 (arrows). FITC, fluorescein isothiocyanate; PE, phycoerythrin.

degree of separation between the CD7⁺ and CD7⁻ T cells varied between cases, creating difficulty in applying objective gates to distinguish these cell populations. For these reasons, quantitative flow cytometry was not considered superior to routine FCIP for assessing the CD4⁺/CD7⁻ T cells.

A global increase in circulating CD4⁺ T cells resulting in a CD4/CD8 ratio of greater than 10 has been proposed by the European Organization for Cancer Treatment and Research and the ISCL as another criterion for the diagnosis of SS.^{18,22} In the SS and the control groups, the CD4/CD8 ratio was calculated by quantitative T-cell subset analysis (Table 5). The median absolute number of circulating CD4⁺ T cells and the median CD4/CD8 ratios were significantly higher in the SS group compared with all control groups. In 5 of 11 SS cases,

the CD4/CD8 ratio exceeded 10, and in an additional 2 cases, the ratios were 9.2 and 9.3. In only 1 SS case was the CD4/CD8 ratio less than 4 (3.2), and this patient had received previous ECP. In contrast, the highest observed CD4/CD8 ratio in all control groups was 4.8, and in all of these groups, the median CD4/CD8 ratio was near 2.5.

Assessment of T-Cell Clonality

T-cell clonality was assessed by TCR PCR and by TCR-V β flow. Clonal TCR PCR gene rearrangements were detected in 10 of 11 SS cases (Table 3). In 1 SS case, the TCR PCR results were equivocal owing to the presence of more than 3 distinct rearrangement peaks; clonality was established in this case by TCR-V β flow. The TCR PCR studies were negative in

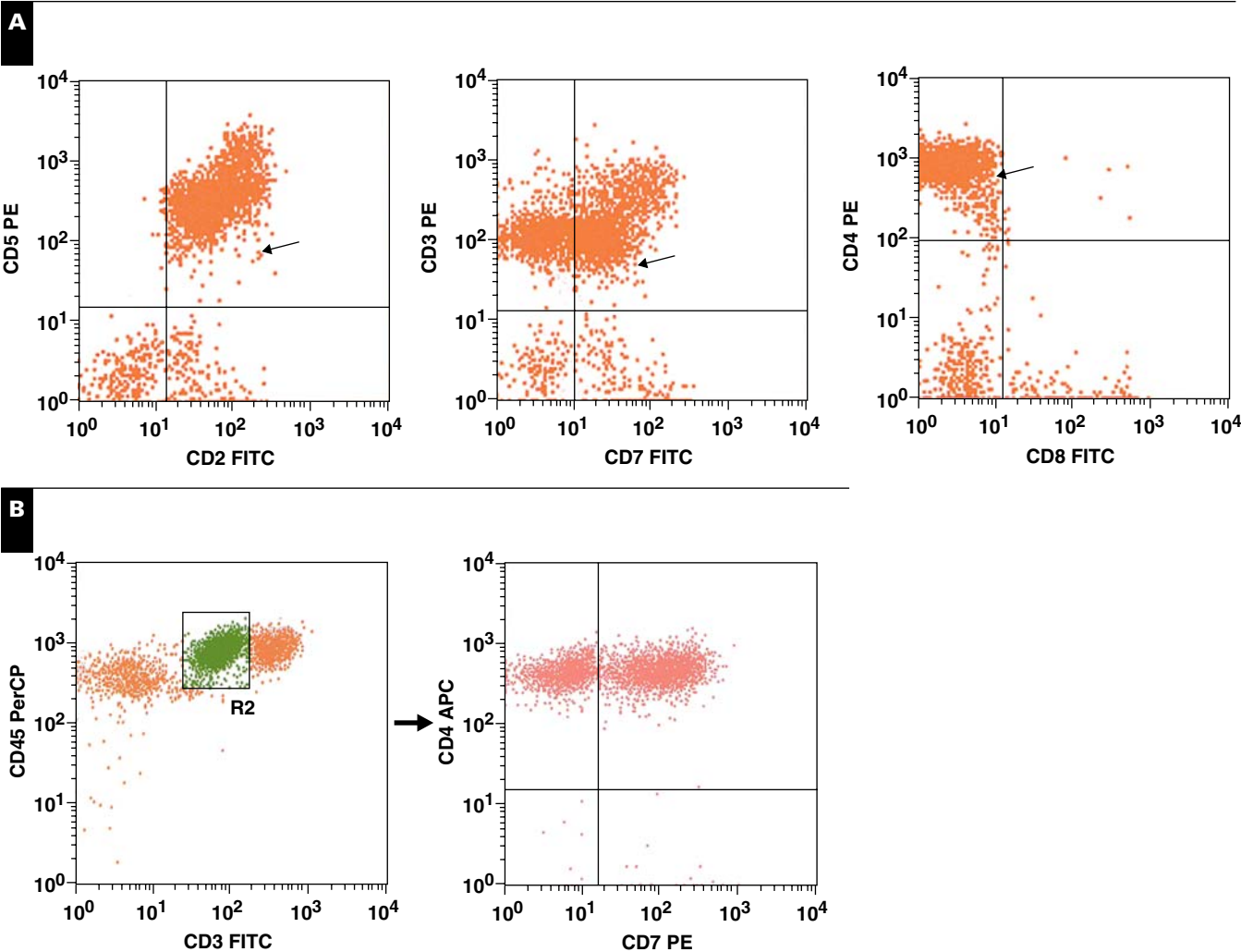


Image 3 Sézary syndrome. **A**, Two-color flow cytometric immunophenotyping revealed the presence of a CD4+ T-cell population with abnormally diminished expression of CD3 and CD7 (arrows). The staining intensity for CD2 and CD5 also seemed slightly decreased. **B**, Four-color flow cytometric immunophenotyping allows for selective gating on the T cells with abnormally diminished CD3 expression. These CD3-dim cells were proven to be CD4+ by this technique and to have partial loss of CD7 with distinct CD7+ and CD7- subsets. APC, allophycocyanin; FITC, fluorescein isothiocyanate; PE, phycoerythrin; PerCP, peridinin chlorophyll-a protein.

Table 3
Peripheral Blood Findings in Sézary Syndrome Cases, Including Estimation of Disease Burden by Manual and Flow Cytometric Methods

Case No.	FCIP	TCR PCR	Sézary Cell Count (/μL)*	CD4 Cell Count (/μL)	CD4 Clonal Cells (%)†	Clonal CD4 Cell Count (/μL)‡
1	Abnormal	Equivocal	935	2,955	78	2,305
2	Abnormal	Clonal	1,360	4,479	74	2,911
3	Abnormal	Clonal	700	1,222	81	990
4	Normal	Clonal	175	210	55	115
5	Abnormal	Clonal	1,140	2,456	88	2,161
6	Abnormal	Clonal	3,670	10,045	93	9,342
7	Abnormal	Clonal	8,170	10,493	90	9,040
8	Abnormal	Clonal	784	1,867	0	0
9	Abnormal	Clonal	14,100	25,877	98	25,340
10	Abnormal	Clonal	1,380	2,074	99	2,053
11	Abnormal	Clonal	5,660	13,810	99	13,671

FCIP, flow cytometric immunophenotyping; PCR, polymerase chain reaction; TCR, T-cell receptor.
* Calculated from the percentage of Sézary cells determined by manual differential times the absolute lymphocyte count.
† As determined by TCR-Vβ flow cytometry.
‡ Calculated from the percentage of CD4 clonal T cells determined by TCR-Vβ flow cytometry times the CD4 cell count determined by T-cell subset analysis.

Table 4
Results of Sequential Analyses in Sézary Syndrome Cases*

Case No.	Interval (d)	Sézary Cell Count, /μL (% Change)	CD4 Cell Count, /μL (% Change)	CD4 Clonal Cells (%)	Clonal CD4 Count, /μL (% Change)
1					
A	0	940	2,955	78	2,305
B†	86	150 (–84)	1,287 (–56)	81	1,042 (–55)
C†	188	90 (–40)	458 (–85)	75	343 (–85)
2					
A	0	1,360	4,479	74	3,314
B†	27	540 (–60)	3,201 (–29)	76	2,432 (–27)
3					
A	0	700	1,222	81	990
B†	77	400 (–43)	605 (–50)	85	514 (–48)
4					
A	0	175	210	55	115
B‡	20	250 (+43)	346 (+65)	66	228 (+98)
5					
A	0	1,140	2,456	88	2,161
B§	28	860 (–25)	2,359 (–4)	89	2,099 (–3)
6					
A	0	3,670	10,045	93	9,342
B†	28	3,138 (–14)	9,896 (–1)	92	9,104 (–3)

* The percentage of change is from day 0 (the A part of each case).
† Received extracorporeal photopheresis.
‡ Received interferon alfa and a topical corticosteroid.
§ Received systemic and topical corticosteroids.

all control groups with the exception of 2 of the reactive cases; in these 2 cases, the results of TCR PCR were interpreted as equivocal owing to the presence of somewhat distinct peaks in a polyclonal background pattern.

TCR- V_{β} flow was performed in combination with antibodies to CD3 and CD8 to allow for specific gating on the CD8+ and CD8– T-cell subsets (Image 4). These studies revealed abnormalities that were diagnostic of or consistent with clonality in the CD8– T cells in 10 of 11 SS cases (Table

3); the 1 case in which TCR- V_{β} flow studies were negative had received previous ECP. In the 5 SS cases with TCR- V_{β} flow diagnostic of clonality, more than 50% of the CD8– T cells reacted with a single TCR- V_{β} antibody; in 4 of the 5, the TCR- V_{β} antibody–positive cells constituted 98% or more of this T-cell subset. No preferential staining for a particular TCR- V_{β} family was seen in this group. In the remaining 5 cases, 75% or more of the CD8– T cells failed to react with any of the TCR- V_{β} antibodies tested, presumably owing to the

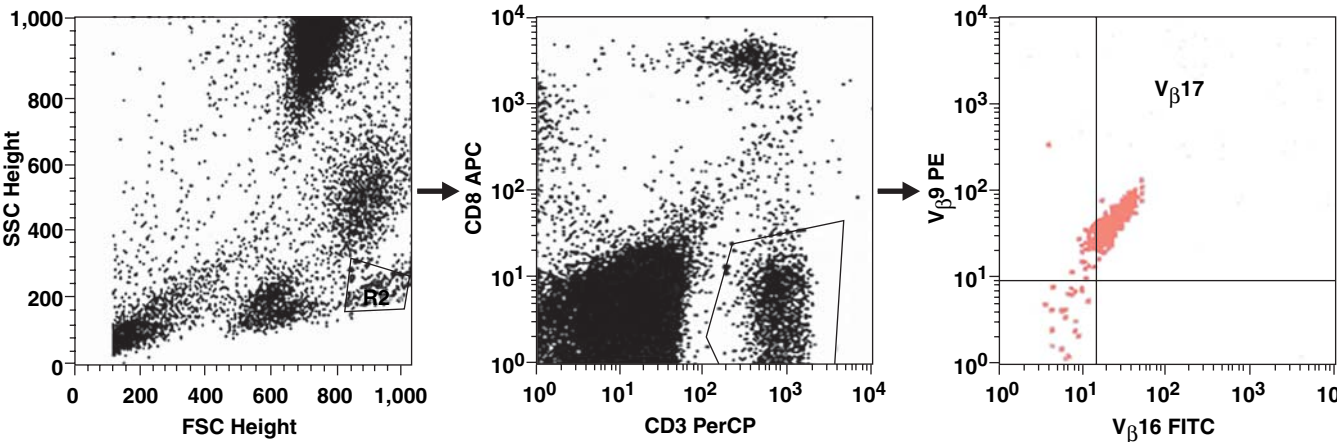


Image 4 T-cell receptor (TCR)- V_{β} flow in Sézary syndrome. The Sézary cells in this case (shown in Image 1) had distinctive forward (FSC) and side light scatter (SSC) characteristics related to their size that allowed for selective gating (left, R2). Additional gating also was done to confirm that the cells were CD3+ and CD8– (center). Essentially all of these cells stained with antibodies to TCR- V_{β} 17 (right), confirming clonality. APC, allophycocyanin; FITC, fluorescein isothiocyanate; PE, phycoerythrin; PerCP, peridinin chlorophyll-a protein.

Table 5
CD4+ T Cells With Loss of CD7 and CD4/CD8 Ratios in Sézary Syndrome Cases and Control Groups*

	Healthy Control Group (n = 11)	Reactive Dermatoses (n = 9)	Sézary Syndrome (n = 11)	Mycosis Fungoides (n = 6)
Percentage of CD4+ T cells with loss of CD7	10.1 (3.6-16.5)	7.0 (2.1-21.0)	33.0 (4.7-96.7)	3.0 (0.8-7.0)
CD4+CD7- ≥40%	0 (0)	0 (0)	5 (45) [†]	0 (0)
CD4 cell count (/μL) [‡]	850 (370-1,150)	920 (320-1,680)	2,950 (210-25,880)	710 (370-1,270)
CD4/CD8 ratio [‡]	2.4 (1.2-3.5)	2.4 (1.2-4.8)	9 (3.2-356)	2.6 (1.5-3.1)
CD4/CD8 ratio ≥10 [‡]	0 (0)	0 (0)	5 (45) [†]	0 (0)

* Data are given as median (range) or number (percentage) unless otherwise indicated.

[†] Sézary syndrome vs reactive dermatoses, $P = .012$; Fisher exact test.

[‡] Calculated by quantitative flow cytometric T-cell subset analysis using an internal bead standard.

expression of a TCR- V_{β} by the clonal CD3+ cells not detected by the antibody reagents. These TCR- V_{β} flow results are considered consistent with clonality because they have been found to be associated universally with clonal *TCR* rearrangements.¹⁵ Clonal TCR PCR gene rearrangements were present in all 5 TCR- V_{β} nonreactive SS cases.

In 1 SS case, T-cell clonality was not detected by TCR- V_{β} flow. In this case, clonality was confirmed by TCR PCR. No abnormalities suggestive of clonality were detected by TCR- V_{β} flow studies in any of the control groups.

Estimation of PB Disease Burden in the SS Cases and Analysis of Sequential Specimens From Individual Patients

The results of PB analyses from each of the initially studied specimens from the SS cases are shown in Table 3. The PB disease burden was calculated from the product of the absolute lymphocyte count and the manually estimated percentage of Sézary cells and the product of the absolute CD4 count and the estimated percentage of clonal T cells in the CD8- compartment as determined by TCR- V_{β} flow. As shown in Table 3, the disease burden estimated by flow cytometry was higher than that estimated by morphologic review in 9 of 11 cases; the exceptions were a case with a very low number of circulating cells (case 4) and a case in which TCR- V_{β} flow failed to detect the clonal population (case 8). These results indicate that in most cases, only a subset of the neoplastic cells are detected by morphologic review.

Serial specimens were obtained in 6 SS cases to assess the usefulness of the various methods in monitoring the number of circulating neoplastic cells; the results are summarized in Table 4. All patients received therapy during the interval between specimen collections; the treatments included ECP (n = 4), systemic interferon alfa and topical corticosteroids (n = 1), and systemic and localized corticosteroids (n = 1).

FCIP analysis of the serially analyzed cases revealed that the patterns of antigen expression by the CD4+ T cells and the proportion of abnormal cells in the CD4- compartment remained essentially unchanged in all cases. The TCR PCR findings also were stable during the study period, including a

case in which the results were equivocal (case 1, Tables 3 and 4). In 3 of 6 cases (cases 1-3, Table 4), a significant decrease in the degree of PB involvement was detected by Sézary cell counting and flow cytometric analysis. As was seen in the initially analyzed specimens, manual Sézary cell counting consistently yielded a lower estimation of PB disease burden than flow cytometry. Despite this, the methods showed reasonably good correlation in their estimation of the percentage of change in the number of circulating abnormal cells.

Previous reports have demonstrated the use of TCR- V_{β} antibodies to monitor a decrement in circulating clonotypic CTCL cells during treatment.^{16,17} Of the 3 cases in which a decrease in the PB disease burden was seen, 1 (case 1, Table 4) contained a TCR- V_{β} antibody-reactive T-cell clone. In the others (cases 2 and 3, Table 4) the clonal T cells failed to react with any of the antibodies tested, and, therefore, the tumor burden had to be estimated indirectly by documenting the number of TCR- V_{β} antibody-negative T cells. In both of these cases, the TCR- V_{β} flow-abnormal cells as a proportion of the total CD3+ T cells decreased commensurately with the Sézary cell and CD4 counts. However, when the CD4- T-cell compartment was analyzed selectively, the proportion of cells expressing the clonotypic TCR- V_{β} in this compartment remained essentially unchanged (Table 4). The propensity of the neoplastic cells to persistently constitute the bulk of the CD4 compartment despite the drop in PB disease burden also is reflected by the nearly identical percentage of decrease seen in the total CD4 and clonal CD4 compartments. This finding also is supported by the FCIP results noted in that the proportion of abnormal cells in the CD4 compartment remained unchanged by this method. Similar results were obtained in the cases in which there was relatively little change in the Sézary cell and CD4 counts.

Discussion

At a recent ISCL conference held to gain consensus on the defining features of SS, it was concluded that quantification of the circulating tumor cells was clinically relevant.¹⁸ In

the present study, PB specimens from patients with SS and a number of control groups were evaluated prospectively by morphologic review, flow cytometry, and molecular genetic methods to compare these modalities in accurately detecting and quantifying the circulating neoplastic cells. The results then were compared with the diagnostic criteria for SS proposed by the ISCL.

A potential drawback of this study design was that, given the rarity of SS, many of the patients (7 of 11) had received previous therapy. However, when the previously treated and untreated cases of SS were analyzed separately, the fractions of cases in each group that met the various ISCL criteria were similar, although the number in each group was insufficient to achieve statistical significance (data not shown). Furthermore, when the findings in our group of patients with SS are compared with other studies (summarized by Vonderheid et al¹⁸), the proportion of cases meeting most of the proposed ISCL criteria is in agreement with previous reports. The one exception to this was the number of cases with a CD4/CD8 ratio of 10 or more (45%), which was slightly lower than that expected based on published literature. Because 4 of the 6 SS cases that failed to reach this criterion had been treated, this may reflect a decrease in the number of circulating cells owing to therapy. However, 2 of the cases were near this cutoff (ratio, >9), and the median CD4/CD8 ratio for the group was high (9). In summary, the attributes of our SS cases seem to be representative of those expected based on the literature despite the heterogeneity regarding previous therapy.

The presence or absence of Sézary cells generally is not regarded as a disease-specific finding because small Sézary cells have been described in PB smears from patients with benign dermatoses and from healthy people.^{4,7,20} In keeping with these reports, Sézary cells were identified in all of the groups, including the majority of the healthy control subjects. Sézary cell quantification, using a minimum absolute Sézary cell count of 1,000/ μ L as recommended by the ISCL, greatly improved the specificity of this finding. A proposed alternative criterion of Sézary cells exceeding 20% of the total lymphocytes also was found to have high specificity and to be more sensitive than the numeric Sézary cell threshold in our group of patients with SS.^{20,21} The ISCL expressed reservations about the use of a percentage-based Sézary cell criterion because some florid reactive dermatoses may have a high percentage of small Sézary cells. Although not encountered in the present study, as suggested by the ISCL, other features of malignancy such as an aberrant T-cell phenotype and evidence of T-cell clonality should be sought before small Sézary cells are equated with malignancy.

Although they were encountered relatively infrequently, large Sézary cells (size approximately equal to a normal monocyte) were an SS-specific finding, as previously reported.²¹ Finally, although Sézary cell enumeration can be helpful

in detecting leukemic E-CTCL, this is difficult to perform without adequately trained staff with high competence in peripheral smear evaluation, and, therefore, it might be impractical for many clinical laboratories.

Multicolor FCIP revealed a distinctly abnormal CD4+ T-cell population in 10 of 11 SS cases. Abnormalities in CD7 expression were encountered most commonly, in keeping with the preponderance of the literature. Concerns have been raised about the usefulness of this parameter, however, because changes in T-cell CD7 expression were encountered in the reactive control group in this study and have been described in reactive T-cell expansions.¹³ The ISCL recommended a threshold of greater than 40% CD4+/CD7- T cells for the diagnosis of SS. This is likely to be relatively insensitive, however, because our data and a number of previously published reports suggest that in most cases, only a subset of the circulating neoplastic cells show complete loss of this antigen.²³

Other immunophenotypic abnormalities were found, specifically in the CD4+ T cells of SS cases, including diminished expression of CD2 and/or CD3 and abnormally bright expression of CD5. These observations are similar to those recently described by Lima et al.¹¹ Abnormalities such as diminished expression of CD4 compared with normal CD4+ T cells and abnormal loss of CD26 are other attributes described in SS¹²; these were not evaluated specifically in the present study. Regardless, given the immunophenotypic variability between individual SS cases, concurrent analysis of multiple T-cell antigens seems to be superior to the evaluation of a single parameter, such as CD7 expression, in the identification of circulating neoplastic cells.

TCR-V β flow revealed features indicative of clonality in most of the SS cases. A distinct advantage of TCR-V β flow as compared with molecular methods such as TCR PCR is that it allows for the direct assessment of clonality in a specific T-cell population of interest. Another advantage of TCR-V β flow is that it provides rapid results in comparison with TCR PCR and Southern blot because both methods require DNA isolation.

There are, however, shortcomings to TCR-V β flow. Technically, one is dependent on appropriate gating within the laboratory for the clone to be detected. This can be problematic if the attributes of the cells (such as forward and side light scatter) are not typical of lymphocytes, and, therefore, the cells might be excluded inappropriately from the analysis. Also noteworthy is that the available TCR-V β antibodies only detect approximately 70% of the TCR-V β families. Therefore, in some cases (including approximately half of the SS cases in this study), the clonal T cells will be nonreactive and will provide only indirect evidence of clonality. In addition, the TCR-V β flow assay is comparative and requires a preponderance of clonotypic cells for their discrimination from normal T cells,¹⁵ which can lead to false-negative results and might explain our

single SS case in which the results of TCR- V_{β} were negative despite the presence of clonal *TCR* rearrangements shown by PCR analysis.

The sensitivity of TCR γ chain PCR in detecting clonality in SS was similar to that of TCR- V_{β} flow. In 1 case, the TCR PCR results were equivocal owing to the presence of more than 3 distinct rearrangement products. The TCR PCR technique uses a primer mix containing multiple upstream and downstream primers and, therefore, is a seminested technique. As a result, this equivocal banding pattern could be attributable to the multiple amplifications of a single rearranged gene. Alternatively, this banding pattern could be due to the detection of dominant cell populations in the oligoclonal reactive CD8+ T cells that were seen by TCR- V_{β} flow in the present study (data not shown) and described by Lima et al¹¹ in SS.

The inability to confidently identify the cell population harboring a gene rearrangement is a disadvantage of TCR PCR and other molecular techniques such as Southern blot. The sensitivity of TCR PCR can, however, be advantageous in some cases, and, furthermore, the results can be compared directly with those that might have been obtained from involved skin or lymph node specimens to confirm the presence of the same cell population in disparate sites. In summary, TCR- V_{β} flow and TCR PCR seem to be complementary techniques, in which the former may be used as an initial test with a rapid turnaround time and the latter to confirm clonality in cases in which the cells are TCR- V_{β} nonreactive or these flow studies are negative in the presence of other morphologic and/or immunophenotypic features to suggest PB involvement by CTCL.

A primary aim of this study was to compare the various methods in estimating PB tumor burden and monitoring changes during treatment. The number of Sézary cells counted by morphologic review consistently provided a lower estimate of tumor burden than results of flow cytometric analyses, indicating that the former detected only a subset of the abnormal cells, as also described by other groups.²³ Despite this, these methods showed a surprisingly good correlation in estimating the degree of change in the number of circulating abnormal cells in serially analyzed cases. The high level of technical competence required for manual Sézary cell enumeration, however, and the propensity of this method to identify only a subset of the neoplastic cells likely makes use of this method for monitoring changes in disease burden impractical.

FCIP studies in serially analyzed specimens documented that the immunophenotypic features of the CD4+ T cells and the proportion of abnormal cells in the CD4 compartment were stable regardless of changes in tumor burden. These FCIP findings are in keeping with a report by Washington et al,²⁴ in which it was shown that FCIP could be used to monitor response to therapy. This approach has value; however, it

might be impractical in some cases, particularly if the immunophenotypic abnormalities are not sufficiently distinctive to allow for the discrimination of abnormal cells from normal T cells or if variability of antigen expression by the neoplastic cells in individual cases makes it difficult to formulate a reproducible gating strategy. Many of these shortcomings also apply to TCR- V_{β} flow, and, furthermore, owing to preanalytic cell processing, both flow methods are semi-quantitative. Because the FCIP and TCR- V_{β} flow results indicated that the clonal cells constituted the vast majority of the CD4+ T cells in SS during treatment, we found that CD4+ T-cell enumeration by quantitative flow cytometric subset analysis could be used to accurately monitor changes in PB tumor burden. Advantages of this approach include that it directly provides quantitative results and that the values are obtained through the use of a highly reproducible, operator-independent gating strategy.

A recently published case report documented a decrease in circulating clonotypic TCR- V_{β} antibody-reactive T cells as a proportion of the total CD3+ T cells in a patient with E-CTCL during response to therapy.¹⁷ The use of TCR- V_{β} flow to monitor changes in PB tumor burden also was described by Schwab et al¹⁶ in a study of 7 patients with CTCL with PB involvement. In this study, a decrease in circulating T cells positive for a single TCR- V_{β} was observed in the 2 subjects with a clinical response to treatment. The decrease in TCR- V_{β} antibody-positive cells as a proportion of the total CD4+ cells was described.

We observed similar results, ie, a decrease in TCR- V_{β} flow-abnormal cells was found in a subset of SS cases during treatment. This decrease was seen in the absolute number of abnormal cells and in their proportion relative to the total CD3+ T cells. In contrast with the findings of Schwab et al,¹⁶ however, when the CD8- T cells were analyzed selectively, we found that the proportion of clonotypic cells in this compartment remained unchanged, even in a patient in whom the PB tumor burden was reduced by 85%.

The cause of the discrepancy between these studies is unclear. It is possible that it may be attributable to the gating strategies and/or antibody combinations used. Alternatively, because the length of posttherapeutic evaluation was considerably longer in the study by Schwab et al,¹⁶ it is possible that this greater time interval allowed for reconstitution of the CD4+ T-cell compartment by normal, polyclonal T cells, lowering the proportion of clonotypic TCR- V_{β} antibody-positive cells. It is difficult to discern from these possibilities because precise data about the gating strategies used and changes in the absolute CD4 count during treatment are not provided by Schwab et al.¹⁶

Regardless, our data suggest that CD4+ T-cell assessment by quantitative subset analysis might be sufficient to monitor PB Sézary cell tumor burden during treatment without the

additional expense of FCIP or TCR- V_{β} flow. These latter studies could be used selectively in cases in which the clinical features suggested a possible change in disease biology.

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