

Evidence for Restricted V β Usage in the Leukemic Phase of Cutaneous T Cell Lymphoma

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Antibodies directed against the β chain of the T cell receptor (anti-V β antibodies) are useful to identify the V β repertoire of T cells in various diseases and to quantify numbers of V β -bearing T cells. The goals of this study were to identify V β + cases of leukemic phase cutaneous T cell lymphoma (CTCL) and to compare the percentage of positive calls with other measures of blood tumor burden, i.e., lymphocyte subsets with a CD4 + CD7– and CD4 + CD26– phenotype and Sézary cell counts. Thirty-three of 49 (67%) cases of leukemic CTCL reacted with an anti-V β antibody. When combined with reports from the literature, the frequency of V β 5 (probably V β 5.1) usage was relatively high when compared with V β 2 that is also frequently expressed by normal CD4 + T cells. The percentage of V β + cells correlated to the percentage of CD4 + CD7– and CD4 + CD26– cells for cases in which the neoplastic cells were deficient in expression of CD7 and CD26, respectively, but not the Sézary cell count. We hypothesize that the increased V β 5.1 usage in CTCL may be the result of depletion of V β 2 and other V β -bearing T cells by staphylococcal superantigens prior to neoplastic transformation, resulting in a relative increase in the frequency of V β 5.1 usage in CTCL.

Key words: cutaneous/enterotoxins/lymphoma/Sézary syndrome/superantigens/T cell/T cell receptor beta chain
J Invest Dermatol 124:651–661, 2005

Cutaneous T cell lymphoma (CTCL) is a clonal expansion of skin-associated neoplastic T cells that typically express a T cell receptor (TCR) $\alpha\beta$ + CD3 + CD4 + CD8– immunophenotype (Kim and Hoppe, 1999). The two major clinical subsets of CTCL are mycosis fungoides (MF) and Sézary syndrome (SS). MF is characterized clinically by the sequential progression from patch to plaque to tumor phases, a distinction based on the degree of cellular infiltration at lesion sites in the skin. SS is characterized by generalized erythroderma and “leukemic” blood involvement (Vonderheid *et al*, 2002). SS may develop *de novo* as erythroderma or be preceded by pruritus alone, non-specific dermatitis, or skin manifestations typical for MF.

Recently, the International Society for Cutaneous Lymphomas (ISCL) proposed five hematologic criteria to define leukemic blood involvement that may occur in SS and less often advanced MF, herein referred to as the leukemic phase of CTCL (Vonderheid *et al*, 2002). These are: (1) an absolute Sézary cell count of 1.0 K per μ L or more; (2) an increase in CD3 + or CD4 + T cells in the blood resulting in

a CD4/CD8 ratio of 10 or more; (3) an increase in T cells with an aberrant phenotype; (4) relative or absolute lymphocytosis with molecular genetic evidence of a dominant T cell clone; and (5) a chromosomally abnormal T cell clone in the blood. Since the ISCL criteria were published, two additional hematologic criteria based on the percentage of CD4 + lymphocytes that are deficient in surface expression of CD7 or CD26 have been proposed to represent leukemic involvement, specifically CD4 + CD7– and CD4 + CD26– percentages of 40% and 30% or more, respectively (Bernengo *et al*, 2001; Vonderheid and Bernengo, 2003). Antibodies directed against the alpha or beta chains of the TCR also have been used clinically to quantify neoplastic T cells in the blood (Vonderheid *et al*, 1994; Scala *et al*, 2002; Schwab *et al*, 2002; Ferenczi *et al*, 2003; Michel *et al*, 2003), but the criteria that differentiates leukemic involvement in CTCL from a lesser magnitude of blood involvement or from benign erythrodermas have not been established.

The human TCR beta V region (TRVB) locus comprises about 65 TRVB gene segments located on chromosome 7 at band 7q34. In 1999, under the auspices of the Human Genome Organization (HUGO) Nomenclature Committee, the classification system proposed by Rowen (Rowen *et al*, 1996) was adopted as the standardized nomenclature (for additional information, see IMGT, the international ImmunoGene Tics information system; <http://imgt.cines.fr> (Initiator and coordinator: Marie-Paule Lefranc, Montpellier, France)). Of the TRVB gene elements, 39–47 genes qualify as functional TRVB genes that belong to 23 V β families. The

Abbreviations: AD, atopic dermatitis; CDR3, complementarity determining region 3; CLA, cutaneous lymphocyte antigen; CTCL, cutaneous T cell lymphoma; HLA, human leukocyte antigen; MF, mycosis fungoides; MHC, major histocompatibility complex; SAg, superantigen; SEA-D, staphylococcal enterotoxin A, B, C, and D; SS, Sézary syndrome; TCR, T cell receptor; TRVB, T cell receptor V beta region; TSST-1, toxic shock toxin 1

Studies performed in Philadelphia, PA, USA.

number of functional genes for each V β family ranges from a single gene in 16 families up to five genes in the V β 5 family, six genes in the V β 7 family, and seven genes in the V β 6 family.

V β usage by peripheral T cells is not proportional to the number of functional genes in each V β family, but is determined primarily in the thymus (Sprent and Webb, 1995). Double-positive CD4 + CD8 + thymocytes that receive a low level stimulation mature into CD4 + or CD8 + via stimulation of the TCR in consort with CD4 or CD8 that mediate contact with class II or class I MHC, respectively. Cells that receive no stimulation or a high level of stimulation against self-MHC-peptides undergo clonal deletion; this is the fate of more than 95% of thymocytes. Because of the close similarity between thymic epithelial cells and keratinocytes (Patel *et al*, 1995), the skin has been implicated in peripheral selection of T cells in both atopic dermatitis (AD) and CTCL (Haynes *et al*, 1982; Mastrandrea *et al*, 1998). Post-thymic events such as selective stimulation of certain V β sequences by superantigens (SAg) further modify the V β repertoire of peripheral T cells.

In normal peripheral blood, certain V β families predominate in the T cell repertoire, e.g., V β 2, V β 4, V β 6, and V β 13, whereas others are rarely used, e.g., V β 10 and V β 18 (Choi *et al*, 1989; Gorski *et al*, 1994; Hall and Lanchbury, 1995; Gran *et al*, 1998). It is thought that differences in reactivity between MHC molecules and V β segments in the thymus influences the V β repertoire of CD4 + and CD8 + cells, and that this accounts for the increased expression of V β 2, V β 5.1, V β 6.7, V β 8, and V β 12 on CD4 + cells compared with CD8 + cells (Grunewald *et al*, 1991; Genevée *et al*, 1994; Reed *et al*, 1994; Shigematsu *et al*, 1996; van den Beemd *et al*, 2000).

Commercially available monoclonal antibodies currently cover more than 80% of the TCR V β repertoire (van den Beemd *et al*, 2000). It is now possible for some of these antibodies labeled with different chromophores to be mixed together and used for rapid screening of suspect T cell lymphoproliferations and immunodeficiency (Langerak *et al*, 2001; Pilch *et al*, 2002; Beck *et al*, 2003; Lima *et al*, 2003). Of relevance to this report that deals with the use of anti-V β antibodies to assess V β usage in a CD4 + malignancy, van den Beemd *et al* (2000) found that the frequency of V β 5.1 expression on normal CD4 + T cells (median, 7.5%) is second only to V β 2 (median, 9.2%) followed by V β 13.1-3 (median, 6.1%), V β 17 (median, 5.4%), and V β 8.1-2 (median, 5.1%).

SAg, when presented by MHC class II molecules on accessory or target cells such as keratinocytes (Travers *et al*, 2001), bind to T cells that bear certain V β segments, resulting in stimulation and proliferation of 10 to 100 times the number of CD4 + and CD8 + T cells that can be activated by conventional antigens (Kotzin *et al*, 1993). This produces a polyclonal expansion of different portions of the T cell repertoire that eventually may be followed by anergy and/or deletion of the stimulated cells (McCormack *et al*, 1993; Miethke *et al*, 1995; Renno *et al*, 1995; Sprent and Webb, 1995). In this paper, we present evidence that V β usage in leukemic phase of CTCL is skewed toward V β 5.1, and hypothesize that this biased usage might be the consequence of the relative depletion of other V β -bearing skin-homing T

cells (negative selection) through the sustained action of skin microbial SAg prior to malignant transformation.

Results

The cases were categorized into three groups based on the observed pattern of V β antibody reactivity (Table S1). Group 1 consists of patients whose neoplastic cells reacted to a single V β antibody in the screening panel (defined as 20% or more positive lymphocytes), and this was observed in 21 of the 42 (50%) samples including 5 previously studied patients with a known V β sequence by RT-PCR and corresponding anti-V β antibody reactivity. In one previously studied patient whose cells were shown to be V β 14 positive by RT-PCR and sequence analysis, 59% of the neoplastic cells reacted with the anti-V β 5.1 antibody, IMM157, but not with the anti-V β 14 antibody, CAS1.1.3 (Bigler *et al*, 1996). This demonstrates that false positive reactions may occasionally occur with V β -specific antibodies. The neoplastic cells of the remaining patients reacted with antibodies directed against V β 5 (9 patients), as well as V β 1 (1 patient), V β 3 (2 patients), V β 6.7 (1 patient), V β 8 (1 patient), V β 12 (3 patients), V β 13.6 (2 patients), and V β 23 (1 patient). When combined with the findings of 7 patients previously characterized with anti-V β antibodies (Table S1, additional group), the neoplastic cells of nine of 13 anti-V β 5-positive cases reacted with anti-V β 5.1 antibody (IMMUN157), three cases reacted with an anti-V β 5.2-3 antibody (MH3-2 or 1C1), and the cells of one patient reacted with both anti-V β 5.2-3 (MH3-2) and anti-V β 5.2 (36213).

Group 2 consists of 12 samples that had ambiguous results with reactivity to more than one anti-V β antibody in the screening panel: six cases reacted to two antibodies, five cases reacted to three antibodies, and one case had positive reactions to six antibodies. The appropriate V β segment could be assigned from previous RT-PCR analysis in two patients and repeat testing or studies on different samples clarified the results with three other cases. Thus, the V β expression on the neoplastic cells could be reliably assigned to five cases, but the other seven cases remain uncertain.

It should be noted that 24%–87% of the neoplastic cells from all seven unclassified samples reacted to an antibody with V β 5 specificity (six samples, V β 5.1/clone IMM157; one sample, V β 5.2-3/clone MH3-2), and 40%–74% of the cells from 5 samples also reacted with anti-V β 23 (clone AHUT7). Although non-specific reactivity related to poor preservation of the cells could explain these results (Beck *et al*, 2003), this seems unlikely because other V β antibodies were typically non-reactive. The possibility that the anti-V β 5.1 clone IMM157 used in the screening panel was yielding a falsely positive reaction must also be considered, especially since a high percentage of cases in Group 1 were positive to this antibody. In most instances, however, other antibodies with V β 5.1 specificity confirmed the results. We suspect that the anti-V β 23 clone AHUT7 may have reacted inappropriately under conditions that we used for screening, and that many of the unclassified samples are truly expressing a V β 5.1 segment. Finally, cross-reactivity of these antibodies or co-expression of V β 5.1 and V β 23 (or other V β

segments) by neoplastic cells cannot be excluded, an interesting possibility considering that approximately 1% of normal T cells express two V β chains (Davodeau *et al*, 1995; Padovan *et al*, 1995). Repeat staining using different antibodies, additional samples or molecular analysis will be required to clarify these results.

Group 3 consisted of nine samples that did not react to any of the anti-V β antibodies in the screening panel. In most cases the percentage of anti-V β -positive cells in the screening panel was less than 1%, suggesting that the number of normal V β -bearing cells in the lymphocyte population was depressed by non-reactive neoplastic cells. Included in this group were three samples for which the V β sequence had been identified by RT-PCR. In two of these cases, the cells did not react to the anti-V β antibody corresponding to the known V β segment, as previously observed (Bigler *et al*, 1996). The other case was not tested using the appropriate anti-V β 18 antibody.

V β usage in leukemic phase CTCL. Altogether, including six previously characterized cases with a V β antibody-positive clone, 33 of 49 (67%) of patients with leukemic CTCL studied at our center were identified as having a neoplastic cell population that reacted with an anti-V β antibody. (Table S1). An additional three patients with a known V β sequence by PCT (V β 5.2-3, V β 18, V β 20) but unconfirmed by an anti-V β antibody increases the total percentage of V β -positive clones to 36 (73%). The frequency of V β usage of leukemic phase CTCL was highest for V β 5 (14/36 or 39%) followed by V β 3, V β 12, and V β 13.6 (3 patients each); V β 6.7, V β 8, and V β 23 (2 patients), and single instances of V β 1, V β 7, V β 14, V β 18, V β 20, V β 21.3, and V β 22.

Our findings, when combined with 136 reports from the literature (Table S2; Fig S1), indicate that V β 5 gene segment usage occurs in about 26% (44 of 172) of patients with leukemic phase CTCL followed by V β 8 and V β 13 (about 10% each). The frequency of V β 5 usage in these cases appears to be unusually high when compared with the frequency of V β usage by normal peripheral blood lymphocytes for which the expression of V β 2, V β 4, V β 6, and V β 13 is even more frequent (Robinson, 1992; Gorski *et al*, 1994; Gran *et al*, 1998). Considering that leukemic CTCL is a CD4+ neoplasm and that V β 5.1 and V β 2 are the most frequent V β segments expressed on normal CD4+ cells, the difference between the frequency of V β 5.1 *per se* and V β 2 usage in leukemic cases when both were studied (23 of 124 cases or 19% vs six of 124 cases or 5%, respectively) is noteworthy.

To assess whether the observed frequency of V β usage in leukemic phase CTCL is skewed, the mean percentages of V β expression on normal CD4+ cells as determined by anti-V β antibodies (based on data on 85 normal specimens provided by Beckman Coulter, Fullerton, California) was used to calculate the expected frequency (number of cases) that might be expected if neoplastic transformation occurred randomly within the T cell population (Fig 1). The mean values for V β 5.1, V β 5.2 and V β 5.3; V β 7.1 and V β 7.2; and V β 13.1, V β 13.2, and V β 13.6 were added together for the calculation, whereas V β 4, V β 6, V β 10, V β 15, and V β 19 were excluded. The distribution between observed and expected V β usage was significantly different (Pearson's χ^2

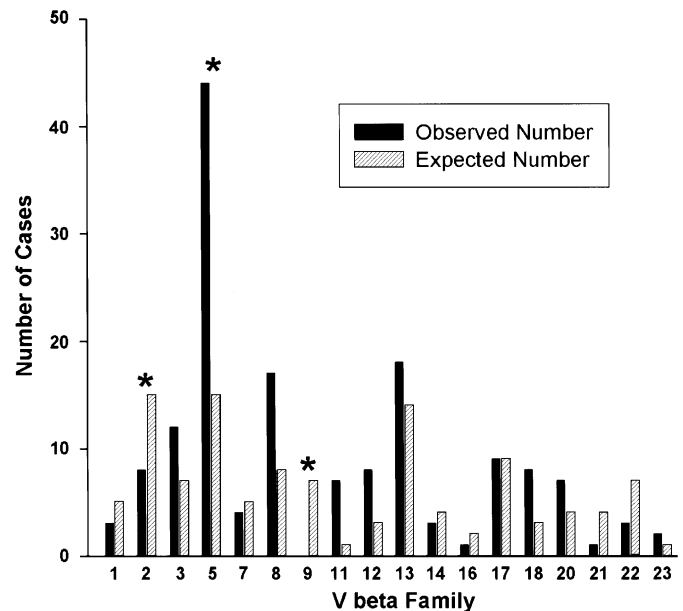


Figure 1

V β 5 expression is increased in leukemic CTCL. The observed frequency of V β usage in patients with leukemic cutaneous T cell lymphoma reported the literature and this series (black bars) is significantly different from the calculated expected frequency derived from mean percentages of normal V β family usage by CD4+ T cells (hatched bars). Asterisks signify the V β families that appear to be significantly increased (V β 5, $p = 0.003$) or decreased (V β 2, $p = 0.027$; V β 9, $p = 0.002$) when tested against other V β segments using Fisher's exact test.

test, $p < 0.001$). The observed frequency of V β 5 was increased and of V β 2 and V β 9 was decreased compared with the expected (Fig 1). Whether V β 5.1 *per se* is significantly increased over the expected could not be addressed, but it should be noted that unlike V β 5.2-3, V β 5.1 usage appears to decrease on normal CD4+ cells with aging (van den Beemd *et al*, 2000). Considering that leukemic CTCL tends to occur in older people and that the distribution of different V β families on CD4+ T cells in normal individuals seems fairly stable throughout life (Cossarizza *et al*, 1995), one might expect an even lower expected frequency of V β 5 usage than was reflected by use in our calculations of all age ranges.

Correlation of V β expression with other markers of blood tumor burden

The percentage of V β -positive lymphocytes was correlated to the percentage of CD4+ CD7- and CD4+ CD26- lymphocyte subsets by flow cytometry, and the number of Sézary cells per 100 lymphocytes on blood smears obtained at the same time as the blood sample for antibody studies. (Table S1) Although not specific for neoplastic cells, these parameters have been utilized to measure blood tumor burden in CTCL, and to differentiate erythrodermic CTCL from benign erythrodermas (Bernengo *et al*, 2001; Vonderheid *et al*, 2002; Vonderheid and Bernengo, 2003).

The percentage of V β antibody-positive cells in our patients ranged from 28% to 97% ($n = 33$; median, 67%; mean $\pm$ SD, 65% $\pm$ 21%). The percentage of V β -positive cells followed a normal distribution, suggesting that the sampling was representative (Kolmogorov-Smirnov normality test, $p > 0.2$). The percentage of CD4+ CD7- cells varied from 0.8% to 92.7% ($n = 30$; median, 42%; mean $\pm$ SD,

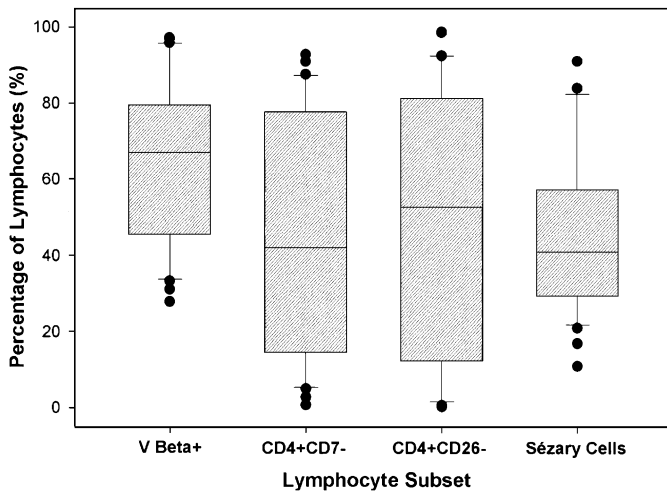


Figure 2

Comparison of V β positive cells to other measures of blood tumor burden. The median percentage (horizontal line) of V β + cells (67%) is significantly higher than the median percentage of CD4 + CD7– cells (42%) and Sézary cells (41%), but was not different than CD4 + CD26– cells (53%). The box represents the 25th and 75th percentiles and the error bars are the 10th and 90th percentiles.

45% $\pm$ 32%), the percentage of CD4 + CD26– cells varied from 0.3% to 98.7% (n, 30; median, 53%; mean $\pm$ SD, 51% $\pm$ 34%), and the percentage of Sézary cells on smears ranged from 11% to 91% (n, 33; median, 41%; mean $\pm$ SD, 46% $\pm$ 22%). The median values for the percentage of V β + cells was significantly higher than the percentage of CD4 + CD7– cells (M-W, $p=0.02$) and the Sézary cell count (M-W, $p=0.002$), but not for CD4 + CD26– (M-W, $p=0.160$) (Fig 2). The higher values for V β + cells compared with the percentage of CD4 + CD7–, but not CD4 + CD26– cells, could reflect the contribution of CD8 + V β + cells in the sample (unlikely due to the selection of cases with high CD4/CD8 ratios) (Lima *et al*, 2003), and/or the fact that CD7 is less likely to be deficient on neoplastic T cells than CD26 (most likely explanation) (Bernengo *et al*, 2001; Vonderheid and Bernengo, 2003). The difference between the percentage of V β + cells and the Sézary cell count likely reflects the fact that Sézary cells tend to be undercounted by visual inspection on smears, especially when they are of small size (Heald *et al*, 1994).

A weakly positive, but statistically significant correlation was found between the percentage of V β + cells and CD4 + CD7– cells in these samples (n, 30; $\rho=0.370$, $p=0.044$), but not the percentage of CD4 + CD26– cells (n, 30; $\rho=0.275$, $p=0.140$). (Fig S2) These correlations, however, are misleading because the neoplastic cells of CTCL are not always deficient for CD7 or CD26 expression. Indeed, CD7 will be expressed at normal or near normal levels on the neoplastic cells in about 30%–40% of patients with leukemic phase CTCL (Vonderheid *et al*, 2001; Vonderheid and Bernengo, 2003), and the same applies for CD26 albeit for a smaller proportion of cases (Bernengo *et al*, 2001; Vonderheid and Bernengo, 2003). Thus, the strength of the correlation of V β positivity with either the CD4 + CD7– or CD4 + CD26– subset in any series of leukemic patients will be influenced by the number of cases in the series with neoplastic cells expressing CD7 or CD26, respectively. The admixture of CD7 + and CD7– cases and CD26 + and

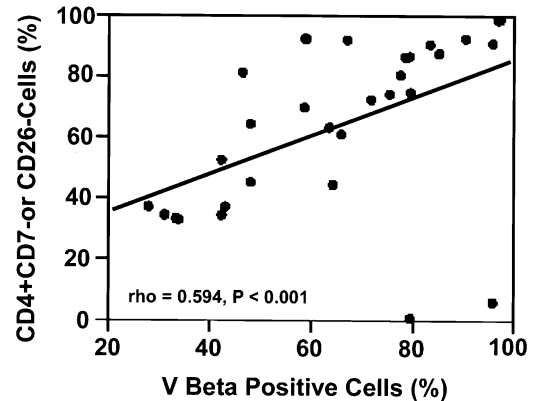


Figure 3

Correlation of V β positive cells to CD4 + CD7– or CD4 + CD26– subsets. The percentage of V β + neoplastic cells correlates well with the maximum percentage of CD4 + CD7– or CD4 + CD26– cells, whichever is greatest (n = 30, $\rho=0.594$, $p<0.001$).

CD26– cases may also underlie the observed lack of correlation between the percentage of CD4 + CD7– and CD4 + CD26– cells (n, 30; $\rho=0.231$, $p=0.218$).

In this series, CD7 and CD26 were expressed on more than 50% of CD4 + lymphocytes in 14 of 30 (47%) and eight of 30 (27%), respectively. To be clinically useful for distinction from normal cells, most of which express CD7 and CD26 (Bernengo *et al*, 2001; Jones *et al*, 2001), a considerable proportion of the neoplastic cells must show deficient expression of CD7 or CD26. If the 16 cases with deficient expression of CD7 on CD4 + cells are analyzed, then a stronger correlation between V β + cells and CD4 + CD7– becomes apparent ($\rho=0.675$, $p=0.004$). Similarly, the correlation between V β + cells and CD4 + CD26– for the 22 cases in which CD26 is expressed on less than 50% of the CD4 + cells now becomes highly significant ($\rho=0.824$, $p<0.001$).

Another way to express this is to correlate V β + cells to either CD4 + CD7– or CD4 + CD26– cells, whichever is highest, based the assumption that CD26 may be deficient on CD7 + cases and vice versa. (Fig 3) Only two of our patients had neoplastic cells that expressed both CD7 and CD26 on most cells. If such an analysis is performed on our samples, a strong positive correlation is observed (n, 30; $\rho=0.594$, $p<0.001$). These observations demonstrate that V β expression on neoplastic cells correlates well with other antibody-based measures of blood tumor burden in CTCL, provided the appropriate phenotype of the neoplastic cells is used, i.e., cells that are deficient for CD7 or CD26.

Finally, no correlation was found between Sézary cell counts and the percentages of V β -positive cells (n, 33; $\rho=0.122$, $p=0.496$), CD4 + CD7– cells (n, 30; $\rho=0.175$, $p=0.350$), nor CD4 + CD26– cells (n, 30; $\rho=0.148$, $p=0.432$). Even if the maximum percentage of CD4 + CD7– or CD4 + CD26– for each case was used, the correlation with Sézary cell counts remained poor (n, 30; $\rho=0.264$, $p=0.157$). A weak correlation between Sézary cell counts and the percentage of V β -positive cells was also found for 44 patients with leukemic CTCL reported in the literature ($\rho=0.093$, $p=0.548$) (Heald *et al*, 1994; Gorochoff *et al*, 1995; Bernengo *et al*, 2001; Rappl *et al*, 2001; Scala *et al*, 2002; Michel *et al*, 2003). These observations indicate

that Sézary cell counts are a poor measure of and often underestimate blood tumor burden in patients with the leukemic phase of CTCL.

Discussion

Our studies of leukemic phase CTCL with anti-V β antibodies indicate that V β usage may be skewed toward V β 5, particularly V β 5.1. Of 32 V β antibody-positive cases in this series, the neoplastic cells from 9 (28%) reacted with the anti-V β 5.1 clone IMMUN157, and 4 additional cases reacted with anti-V β 5.2-3 clone MH3-2. Other frequently positive reactions were to anti-V β 3.1, V β 12, and V β 13.6 (3 cases each).

One must first consider whether the high frequency of V β 5.1 could be a spurious result because of technical reasons or a biased selection of patients. Although we do not think this is likely, it is noted that the frequency of V β 5 usage in our series (40%) was significantly higher than what has been reported for leukemic CTCL in the literature (Fisher's exact test; $p=0.019$). Nevertheless, in reported cases of leukemic CTCL, V β 5 also was used more commonly than other V β segments (30 of 138 or 22%) followed by V β 8 (11%), V β 13 (11%), and V β 17 (7%) (Fig S1). V β 5.1 positivity also was about twice as frequent as V β 5.2-3 in our patients, but the ratio was equal in cases reported in the literature (13 cases bearing V β 5.1 vs 13 cases bearing V β 5.2 or V β 5.3 segments; four cases unknown); this difference was not significant (exact χ^2 test, $p=0.233$). Because certain anti-V β antibodies may react in a less restricted fashion, e.g., the anti-V β 8 clone MX11 (Jack and Carrel, 1990), consideration must also be given to the possibility that the anti-V β 5.1 clone IMMUN157 used in our studies was also prone to inappropriate reactivity. We do not think this is likely because more V β 5.1+ cases reported in the literature reacted to the anti-V β 5.1 clone LC4 than clone IMMUN157. At this time molecular analysis of V β usage are not available to confirm these results.

Immunohistochemical studies on lesional skin of CTCL have shown that a dominant neoplastic clone is often difficult to demonstrate with an anti-V β antibody. Of 11 series reported in the literature, (Jack and Carrel, 1990; O'Grady *et al*, 1990; Ralfkiaer *et al*, 1991; Bagot *et al*, 1992; Bahler *et al*, 1992; Boehncke *et al*, 1992; Gilks *et al*, 1992; Hunt *et al*, 1992; McHenry *et al*, 1994; Finn *et al*, 1996; Potoczna *et al*, 1996), a V β + clone was detected in lesional skin of only 33 of 173 (19%) patients with MF and seven of 29 (24%) patients with SS. Most of these studies were, however, performed with a limited panel of 5 or 6 anti-V β antibodies that were available at the time, e.g., V β 3.1 (clone 8F10), V β 5a/5.2-3 (clone 1C1), V β 5b/5.3 (clone W112), V β 6a (clone OT145), V β 8a (clone 16G8), and V β 12a (S511) from T Cell Diagnostics, Woburn, MA. Anti-V β 5.1 antibody (clone LC4) was included in the diagnostic panel in five of these series (O'Grady *et al*, 1990; Bahler *et al*, 1992; Hunt *et al*, 1992; Finn *et al*, 1996; Potoczna *et al*, 1996), and only the study of Potoczna *et al* (1996) used a broad panel of 21 antibodies that covered most of the T cell repertoire. One study reported a high frequency of V β 8 restriction in nine of 16 cases of MF using clone MX11 (Jack and Carrel, 1990),

but to our knowledge such a restricted V β repertoire was never confirmed in other studies with this antibody nor with different anti-V β 8 clones 16G8 or MX6. Another factor is that the V β antibody may not react appropriately to the V β segment. For example, Bahler *et al* (1992) found the expected positive antibody reaction in only three of nine cases in which neoplastic cells were V β 5-, V β 6-, V β 8-, or V β 13-positive by PCR analysis. False-negative reactivity to anti-V β antibodies also has been observed when neoplastic T cells in the blood of SS were studied by flow cytometry (Gorochov *et al*, 1995; Bigler *et al*, 1996). Thus, the low detection rate of V β + clones in reported studies is related in part to the limited number of available antibodies used in the panel, the relatively low numbers of neoplastic cells in minimally infiltrated lesional skin relative to the reactive cells in the infiltrate, the failure of the particular antibody to react appropriately to the V β segment demonstrated by molecular analysis, as well as failure of certain antibodies to react using immunohistochemical techniques even when combined with antigen retrieval.

Despite these confounding factors, of the reported 40 V β antibody-positive cases of MF/SS in the skin, reactivity against V β 8 was by far the most frequently positive clone (19 cases including nine cases reported by Jack and Carrel (1990) followed by V β 5 (12 cases). If one considers only the five series in which V β 5.1 antibody was included in the screening panel (O'Grady *et al*, 1990; Bahler *et al*, 1992; Hunt *et al*, 1992; Finn *et al*, 1996; Potoczna *et al*, 1996), then a V β 5.1+ neoplastic clone was identified in eight of 21 (38%) of cases that reacted with an anti-V β antibody. These observations suggest that V β 5.1 usage may be more frequent in lesional skin of CTCL than generally appreciated. Conversely, molecular analysis of V β expression in the skin of MF/SS revealed only two V β 5.1+ clones in 37 cases studied with multiple V β family probes, and none of the 15 skin samples of SS were positive for V β 5 (Bahler *et al*, 1992; Jackow *et al*, 1997; Thor Straten *et al*, 1998).

The reason for the apparent discrepancy in V β frequencies between immunohistochemistry and molecular genetic analysis results on skin specimens and between skin and blood samples is unclear. It seems conceivable that neoplastic T cells in advanced CTCL might lose surface molecules that are involved in homing into the skin such as cutaneous lymphocyte antigen (CLA) and this would decrease the number of neoplastic cells in the skin relative to the blood (Heald *et al*, 1993). Indeed Scala *et al* (2002) found that CLA was expressed on only a minority of V β + neoplastic cells in the blood of patients with advanced SS. Alternatively, V β expression in the skin may be difficult to assess because the density of neoplastic cells in the skin of SS is often quite low compared with the non-neoplastic reactive cell population that itself could show skewed V β usage due to the influence of bacterial SAg (Jackow *et al*, 1997).

Several possibilities might account for the observed increase usage of V β 5.1 in CTCL. The first possibility is that the frequent V β 5.1 usage simply reflects the fact that V β 5.1 is expressed to a greater degree by CD4+ than CD8+ T cells (Grunewald *et al*, 1991; Genevée *et al*, 1994; Reed *et al*, 1994; Shigematsu *et al*, 1996; van den Beemd *et al*, 2000). This, however, cannot be the entire explanation

because V β 2 segments are expressed to an even greater degree by CD4 $^{+}$ cells and these segments are infrequently identified in leukemic CTCL. Another possibility is that V β 5.1-bearing T cells may be inherently more prone to undergo malignant transformation relative to other V β families. The V beta locus on the long arm of chromosome 7 at band 7q34 is rarely involved in CTCL, but an oncogenic virus might conceivably use V β 5.1 sequence for entrance into the cell. A third possibility to consider is whether neoplastic V β 5.1 $^{+}$ T cells might be more responsive to mitogenic stimulation *in vivo* than other V β -bearing cells and therefore more likely to expand and present as leukemic CTCL. Although there is currently no evidence that this occurs, it should be noted that V β $^{+}$ Sézary cells can proliferate in response to staphylococcal SAg via the relevant V β segment (Tokura *et al*, 1992; Tokura *et al*, 1995). These observations demonstrate that staphylococcal proteins can induce neoplastic cells to proliferate, and because keratinocytes in CTCL express class II receptors (Tjernlund, 1981; Stevens *et al*, 2003) proliferation of neoplastic T cells stimulated by endotoxins that bind to the V β 5.1 segment could occur in the skin microenvironment. Finally, there is clinical evidence that the skin manifestations of SS can be ameliorated by topical or systemic antibiotic therapy (Tokura *et al*, 1995; Jackow *et al*, 1997). A fourth possibility is that microbial SAg (or antigen) might quantitatively affect the pool of T cells from which a V β 5.1 $^{+}$ neoplastic emerges when malignant transformation occurs. This possibility seems to us to be more likely than the others and will be developed as a hypothesis in some detail (Fig 4).

Hypothesis: bacterial superantigens play a selective role in the development of CTCL Considerable evidence

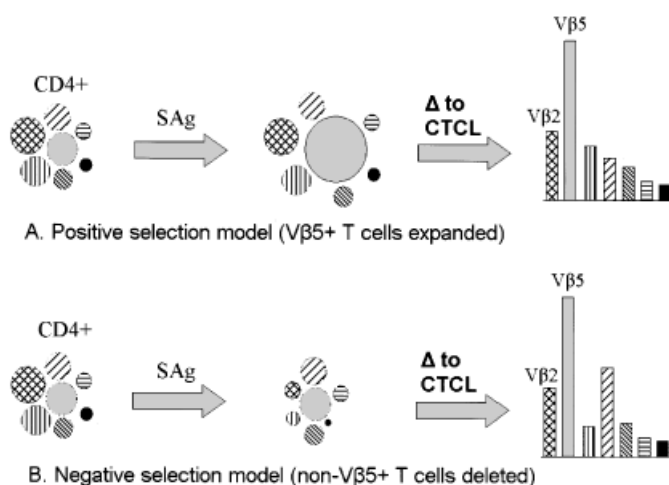


Figure 4
Bacterial superantigens might increase the frequency of V β 5 usage in leukemic cutaneous T cell lymphoma via two hypothetical mechanisms. CD4 $^{+}$ skin-homing T cells expressing V β 5 segments, represented as a solid gray circle among other V β segments, could proliferate and expand in response to superantigenic stimulation relative to other T cells (panel A). Conversely, superantigen-sensitive V β -bearing cells such as V β 2, shown as a cross-hatched circle, could undergo activation-induced apoptosis and depletion in response to superantigenic stimulation, leaving behind a relative increase in V β 5 and other unresponsive T cells (panel B). When neoplastic transformation occurs, a relative increase of V β 5 usage would occur with either model.

has accumulated to indicate that microbial SAg can modulate V β expression of T cells in the skin and play an important role in the pathogenesis of various skin diseases (Skov and Baadsgaard, 2000; Yarwood *et al*, 2000). For example, the skin of patients with AD is often colonized by enterotoxin-producing strains of *Staphylococcus aureus*, and these superantigenic toxins are capable of skewing the V β subfamily repertoire in skin-homing T cells as well as increasing expression of the skin-homing receptor, CLA (Leung *et al*, 1995; Neuber *et al*, 1996; Torres *et al*, 1998; Dworzak *et al*, 1999; Strickland *et al*, 1999; Bunikowski *et al*, 2000; Davison *et al*, 2000; Skov *et al*, 2000; Zollner *et al*, 2000; Breuer *et al*, 2002). Even with healthy individuals, molecular studies have shown differences in V β family expression in skin samples compared with the blood, suggesting an effect of the microenvironment of the T cell repertoire (Dunn *et al*, 1993; Ahangari *et al*, 1996; Menssen *et al*, 2000). Although these studies did not agree on which V β expression predominates in normal skin, V β 5 usage in general and V β 5.1 in particular was underexpressed relative to the blood. This observation indicates that CTCL is unlikely to arise from the indigenous T cell population in the skin.

Because some cases of CTCL are preceded by a chronic dermatitis or AD (Rajka and Winkelmann, 1984; Abel *et al*, 1986; van Haselen *et al*, 1999), it seems plausible that the increased usage of V β 5.1 in leukemic CTCL may be because neoplastic transformation is more likely to involve a subset of V β 5.1 $^{+}$ skin-homing T cells that is more numerous relative to other V β segments. Perhaps bacterial SAg play a role in the selection of V β 5.1 neoplastic clones. In this regard, Linnemann *et al* (2004) recently characterized the TCR γ gene sequences of V β 5.1-expressing T cells isolated from skin lesions of one patient with V β 5.1 $^{+}$ plaque phase MF. About 60% of the V β 5.1 $^{+}$ cells in the epidermis belonged to the neoplastic clone whereas about 67% of the V β 5.1 $^{+}$ cells in the dermis were different from the neoplastic clone and were polyclonal in origin. A staphylococcal infection was also present, but details were not provided. This study suggests that the neoplastic cells in this case could have arisen from an increased number of polyclonal V β 5.1-bearing T cells in the skin.

In support of the hypothesis that the number of V β 5.1 $^{+}$ T cells is often increased in dermatitis such as AD, Potoczna *et al* (1996) found V β 5.1 (clone LC4) expression by immunohistochemistry to be common in eczematous skin as well as CTCL. In addition, Torres observed a significantly increased percentage of CLA $^{+}$ V β 5.1 $^{+}$ T cells in children with AD compared with normal controls (Torres *et al*, 1998). Most other studies that evaluated the V β repertoire in AD have, however, not shown an increase in V β 5.1 in the skin (Neuber *et al*, 1996; Yudate *et al*, 1996; Bunikowski *et al*, 2000), nor blood of AD patients (Strickland *et al*, 1999; Bunikowski *et al*, 2000; Davison *et al*, 2000) compared to normal controls, and Neuber *et al* (1996) actually found a significantly decreased percentage of V β 5.1 $^{+}$ T cells in the skin of AD. Therefore, it is possible but probably unlikely that the predilection for neoplastic cells to express V β 5.1 in leukemic CTCL is the result of malignant transformation of normal skin-homing V β 5.1 $^{+}$ T cells that have expanded as a result of the direct effect of bacterial SAg. The possibility

of an antigen-specific expansion of V β 5.1+ cells cannot, however, be excluded.

If staphylococcal or other microbial SAg play a significant role in the selection of V β usage in CTCL, then theoretically this might occur as a result of either positive or negative selection forces on skin-homing T cells. (Fig 4) With the positive selection (expansion) model, exposure to microbial SAg *in vivo* stimulates relevant V β -bearing T cells to proliferate, causing a polyclonal expansion of responsive cells (Linnemann *et al*, 2004). To explain the increased usage of V β 5.1 in CTCL, it is necessary to demonstrate that SAg capable of stimulating T cells through V β 5.1 are often present in the skin microflora and that V β 5.1-bearing T cells are disproportionately expanded in such patients. With the negative selection (deletion) model, the effect of the microbial SAg might be to delete V β -responsive T cells through activation-induced apoptosis, leading behind a small proportion of unresponsive (anergic) cells and T cells that bear V β segments unresponsive to the SAg. This model presumes that organisms capable of producing V β 5.1-stimulating SAg would be less abundant on the skin compared with other microorganisms that release SAg and that T cells bearing V β 5.1 and other segments are increased in the skin relative to T cells known to respond to SAg. Assuming that neoplastic transformation occurs randomly within the skin-homing T cell population, then the increased usage of V β 5.1 in CTCL could be explained with either model.

The most extensively studied staphylococcal toxins with SAg capacity that colonize skin disorders like AD are the staphylococcal enterotoxins A, B, C, and D (SEA–D) and toxic shock toxin 1 (TSST-1). Toxin-producing strains are isolated in 50%–65% of patients, and a single strain of *S. aureus* may produce several toxins. Moreover, bacterial SAg may stimulate T cells through more than one V β segment. A compilation of enterotoxins produced by *S. aureus* isolated from the skin lesions of AD from nine series in the literature indicated that the frequency was highest for SEB (32%) followed by SEA (26%), SEC (17%), TSST-1 (16%), and SED (9%) (Hoeger *et al*, 1992; Leung *et al*, 1993; McFadden *et al*, 1993; Akiyama *et al*, 1996; Nomura *et al*, 1999; Bunikowski *et al*, 2000; Zollner *et al*, 2000; Breuer *et al*, 2002; Mempel *et al*, 2003). In terms of the major V β families expressed by normal peripheral T cells, TSST-1 can stimulate V β 2-bearing cells, SEC3 can weakly stimulate V β 5.1-bearing cells, SEC2 and SEC3 can stimulate V β 13.1-3-bearing cells, and SEB and SEC2 can stimulate V β 17-bearing cells (Kotzin *et al*, 1993; Leung *et al*, 1995; Davison *et al*, 2000; Petersson *et al*, 2003; Proft and Fraser, 2003). It therefore seems unlikely that any of these common staphylococcal SAg play much of a role in a positive selection of V β usage in CTCL. V β 5.1-bearing T cells may, however, be activated by SEE, which also stimulates V β 8+ T cells, as well as by several members of a recently characterized toxin-producing staphylococcal family associated with the enterotoxin gene cluster (e.g., SE I, K, L, and Q) that has been identified on nearly 50% of isolates from AD (Orwin *et al*, 2001; Orwin *et al*, 2002; Mempel *et al*, 2003; Orwin *et al*, 2003). Furthermore, some species of *Streptococcus pyogenes* and *Candida albicans* produce SAg capable of stimulating V β 5.1-bearing T cells (Walsh *et al*, 1996; Proft and Fraser, 2003). Therefore, the microflora in CTCL could

potentially harbor organisms that could expand V β 5.1+ T cells prior to neoplastic transformation, but direct evidence for this is currently lacking.

Jackow *et al* (1997) correlated bacterial toxin production by *S. aureus* and V β usage in CTCL including 14 patients with SS. The toxins produced by these organisms were characterized in 16 isolates, and in nine cases more than one toxin with superantigenic activity was identified, a result similar to patients with AD. Six isolates had evidence of TSST-1, and the authors correlated this to overexpansion of V β 2+ T cells in the skin and/or blood. It seems possible that the V β 2+ cells in these cases represented a polyclonal expansion of reactive cells rather than an expanded neoplastic population. It also should be noted that V β expression in skin from healthy controls often achieved similar levels of expression, rendering interpretation of these results problematic (Musette and Bachelez, 1997). Nevertheless, this study provides no evidence that V β 5+ T cells are preferentially expanded in the lesional skin of CTCL.

A major argument against the SAg-mediated positive selection hypothesis as stated above is the fact that V β usage by neoplastic cells is not increased for V β segments that are stimulated by commonly occurring staphylococcal SAg, e.g., V β 2 via the action of TSST-1 or V β 3 or V β 17 via the action of SEB, etc. In fact, considering that V β 2 is one of the most frequently expressed V β segments by normal blood T cells and that V β 2 stimulation by TSST-1 occurs in AD, it is surprising that V β 2 is not expressed at a higher frequency in leukemic CTCL. In addition, if a SAg were implicated in a polyclonal expansion of responsive T cells in CTCL prior to malignant transformation as suggested by the work of Linnemann *et al* (2004), then there might also be an increase in CD8 cells bearing the same V β segment as the neoplastic cells. In this regard, Lima *et al* (2003) observed one or more expansions of a V β segment for CD8+ T cells in 14 of 16 patients with a CD4+V β + SS. In three cases where analysis of V β expression was performed on both the neoplastic CD4+ and normal CD8+ cells, the V β usage differed between the subsets. This finding argues against the positive selection model by SAg. It should, however, be noted that the effect of bacterial SAg on CD8+ cells is less profound than on CD4+ cells and this could produce a differential effect on V β usage *in vivo* (Kawabe and Ochi, 1991).

The alternative hypothesis, i.e., the negative selection by SAg prior to neoplastic transformation, proposes that chronic exposure to these microbial SAg might act to deplete T cells bearing V β 2, V β , or V β 17, accounting for the unexpectedly low frequency of usage of these V β segments in CTCL, and because V β 5.1 is not affected by TSST-1 and SEA–D, subsequent malignant transformation would be more often involve unaffected V β 5.1+ cells. This would explain the disproportionately high frequency of V β 5.1 *vis-à-vis* V β 2.

Support for this hypothesis comes from studies in mice that were injected with SE. Single doses result in an initial rise in the proportion of reactive V β cells followed by a dramatic decline, mediated mostly by Fas–FasL-induced apoptosis (McCormack *et al*, 1993). If administered repeatedly in low doses, almost total depletion of target cells occurred in the absence of apparent prior proliferation. Recovery gradually occurred from newly generated cells

in the thymus in the absence of enterotoxin, and a proportion of the target V β + cells became anergic to further stimulation. Some T cells are activated by the SAg in an antigen-specific manner, and repetitive stimulation may eventually result in T cell oligoclonality (Kim *et al*, 2003). In humans, repeated ligation of the TCR or SAg also results in activation-induced cell death (Weber *et al*, 2000). In AD, which serves as a paradigm for the effect of the microbial SAg on skin-homing T cells, a high percentage of CLA + CD45RO + T cells in the peripheral blood are activated and undergoing apoptosis compared with normal controls (Yoshino *et al*, 2000; Akdis *et al*, 2003). T cells with a T_H2 cytokine profile were more resistant to undergoing apoptosis than T_H1 cells (Akdis *et al*, 2003). It is therefore conceivable that chronic exposure to one or more bacterial SAg in the skin may ultimately deplete responsive V β + cells analogous to what is observed in mice.

Recently, blood samples from patients with CTCL have been analyzed by complementarity determining region 3 (CDR3) length spectratyping, also known as immunoscopy (Ingen-Housz-Oro *et al*, 2002; Yawalkar *et al*, 2003). This is an RT-PCR-based method that is semi-quantitative and highly sensitive. A recent study with the immunoscope technique on patients with CTCL without overt blood involvement (all with blood CD4/CD8 ratios less than 10) showed multiple monoclonal and oligoclonal CDR3 spectratype patterns as well as loss of other V β families including patients with early disease (Yawalkar *et al*, 2003). These results suggest a global assault on the T cell repertoire across multiple V β families reminiscent of spectratypes found in advanced human deficiency disease. Although human T cell lymphotropic virus type I has been linked to CTCL (Pancake *et al*, 1995), and this potentially could account for the findings by immunoscopy, it seems possible that sustained superantigenic stimulation of sensitive T cell populations by a microbial antigen might also cause depletion of such cells in the blood. Further investigations on conditions like AD are needed to evaluate this hypothesis.

If a microbial SAg is involved in depleting part of the V β repertoire of skin-homing T cells prior to neoplastic transformation, then this could also explain the association of CTCL with both HLA class I molecules, e.g., HLA-B8 (Dick and Mackie, 1977; Rosen *et al*, 1985), and class II molecules, i.e., HLA-DR (DRB1*11), and HLA-DQB1*03 alleles in MF and SS (Jackow *et al*, 1996; Hodak *et al*, 2001) as well as HLA-DQB1*0502 in SS (Jackow *et al*, 1996). The association with class I HLA alleles and infectious disease have been demonstrated mainly with HLA-B (Cooke and Hill, 2001), and interactions between class I and class II may contribute to disease susceptibility (Harbo *et al*, 2004). It is tempting to speculate that the expression of a particular HLA phenotype on epidermal cells of patients with CTCL might excessively influence the T cell response to staphylococcal SAg through V β sequences as has been reported for streptococcal SAg (Kotb *et al*, 2003).

The possibility that antigenic rather than superantigenic stimulation is responsible for the increase in V β 5.1 usage must also be considered. This possibility can be addressed by sequencing the CDR3 of the TCR to determine if there is a preferential use of any BJ segment for V β 5.1 + cells. In this regard, however, Ingen-Housz-Oro *et al* (2002) found no

common CDR3 motif was found among samples from 15 patients with SS.

The clinical implications of these observations suggest that the V β 5.1 segment might be selected as a target for initial immunotherapy studies using naked DNA vaccination or dendritic cell immunotherapy (Reddy *et al*, 2001). In addition, cytotoxic immune responses against TCR V β epitopes that were induced using V-region-derived peptides from neoplastic CTCL cells have been demonstrated *in vitro* (Winter *et al*, 2003), and the therapeutic effect of extracorporeal photopheresis is mediated in part by an anti-clonotypic response against the V β segment of the TCR (Berger *et al*, 1998). Further studies that target V β 5.1 for immunotherapy would be worthwhile.

Materials and Methods

Forty-nine patients with the leukemic phase of CTCL (19 men, 30 women; median age, 69 y; range, 41–87) were evaluated one by one (E. C. V.) at Hahnemann University (now Drexel University College of Medicine) between 1987 and 2000. Blood samples were obtained and stored with the informed consent of the donor and in accordance with the Declaration of Helsinki protocol. The Institutional Review Board of Hahnemann University approved all described studies.

Statistical analysis The difference in median values for laboratory studies was tested using the nonparametric Mann–Whitney test. The correlation between the percentage of V β + cells and other measures of blood tumor burden was examined using the Spearman rank order correlation test. Fisher's exact test and Pearson χ^2 test for 2 $\times$ 2 tables and R $\times$ C tables, respectively, were used as measures of association among categorized laboratory parameters. The mean percentages of V β expression on normal CD4 + cells as determined by anti-V β antibodies in the IO Test Beta Mark kit was provided by Beckman Coulter (Fullerton, California).

Statistical software used for data analysis was SigmaStat for Windows, Version 3 (Jandel Scientific Software, San Rafael, California), and StatXact-3 (CYTEL Software Corporation, Cambridge, Massachusetts).

Previous observations Initial flow cytometry studies by our group were performed during in the mid-1990s using a limited panel of anti-TCR V β antibodies that were obtained from T Cell Diagnostics (Woburn, Massachusetts) [V β 5a/5.2-3 (1C1), V β 5b/5.3 (W112), V β 5c/5.1 (LC4), V β 6.7 (OT145), V β 8a (16G8), V β 12 (S511), as well as V α 2 (F1)], and Immunotech (Westbrook, Maine) [V β 3 (LE-89), V β 14 (CAS1.1.3), V β 18 (BA62.6), and V β 20 (ELL1.4)]. Of 17 patients with SS and a documented V β -positive clone by RT-PCR, ten expressed a TCR V β segment for which a corresponding anti-V β monoclonal antibody was available to test for reactivity. The V β usage by the neoplastic cells was confirmed with the anti-V β antibody in seven of these cases, whereas the neoplastic cells failed to react in three cases (Bigler *et al*, 1996). This work showed that some anti-V β antibodies fail to react appropriately with cells that have the corresponding V β segment (false-negative reactions), a finding also observed by others (Gorochov *et al*, 1995). In this regard, the neoplastic cells of one patient that did not react with the anti-V β 3 clone LE-89 in the previous screening panel was found in the present study to react with the anti-V β 3 clone CH92. Moreover, subsequent studies (unpublished) of five additional Sézary patients with a V β -positive clone by RT-PCR identified two patients whose neoplastic cells displayed a discordant anti-V β antibody reactivity: one V β 18 by PCR and V β 13 by antibody and the other V β 14 by PCR and V β 5.1 by antibody. The other 3 patients were studied by molecular methods only and expressed V β 13.1, V β 22, and V β 13.2. The available information from these early studies is incorporated into Tables S1 and S2 and Fig S1.

Additional observations Additional characterization of neoplastic cells of CTCL with a large panel of anti-V β antibodies was performed in the flow cytometry laboratory at Hahnemann University between 1999 and 2001. The goal was to identify cases of V β -positive leukemic phase CTCL in our tissue bank that could be used for additional studies with other monoclonal antibodies. Cryopreserved peripheral blood lymphocytes from 47 patients with documented blood involvement were studied with monoclonal antibodies for expression of T cell markers (CD3, CD4, CD7, CD8, CD26) and TCR V β expression using a panel of 20 antibodies conjugated with fluorescein isothiocyanate purchased from Beckman Coulter: TCR V β 1.1-2 (BL37.2), V β 2 (MPB2D5), V β 3 (CH92), V β 5.1 (IMMU157), V β 5.2 (36213), V β 7.1 (ZÖE), V β 8.1-2 (56C5.2), V β 11 (C21), V β 12 (VER2.32.1), V β 13.1 (IMMUN222), V β 13.6 (JU74.3), V β 14 (CAS1.1.3), V β 16 (TAMAYA1.2), V β 17 (E17.5F3.15.13), V β 20 (ELL1.4), V β 21.3 (IG125), V β 22 (IMMUN546), and BD PharMingen (San Diego, California): V β 5.2-3 (MH3-2), V β 9 (AMKB1-2), and V β 23 (AHUT7). To clarify results, additional staining of selective cases was performed with antibodies V β 5a (1C1), V β 5b (W112), and V β 5c (LC4) from BD PharMingen that react against segments V β 5.2-3, V β 5.3, and V β 5.1, respectively. The gate for lymphocytes was based on light scatter, including large lymphocytes when present, and if necessary verified with CD45 and CD14. The percentage of all CD3+, CD3+CD4+, CD3+CD8+, CD4+CD7-, CD4+CD26-, and all V β + cells within the lymphocyte gate was recorded (Table S1).

The complete panel was run on samples from 42 patients including 12 of 19 patients studied earlier with a different V β antibody panel as well as six of 10 patients that were previously reported (Table S1, Groups 1–3). In addition, a limited number of V β antibodies were run on 6 patients with a previously identified V β antibody positive clone as well as a patient with coexisting B-cell chronic lymphocytic leukemia (Table S1, additional cases). At the time of sampling, 46 patients were diagnosed to have SS (generalized erythroderma with leukemic blood involvement), and in eight of these patients, the SS had been preceded by the diagnosis of MF. The other three patients had the cutaneous manifestations of extensive plaque or tumor phase MF. To confirm or clarify uncertain results, the antibody panel was repeated on separate samples from 5 patients (data not shown).

According to the criteria proposed by the International Society for Cutaneous Lymphoma (Vonderheid *et al*, 2002), all patients fulfilled at least one of the hematologic criteria for leukemic involvement (B2 blood rating). Specifically, 31 patients had an absolute Sézary cell count of 1.0 K per μ L or more, 35 patients had an increase in CD4+ cells resulting in a CD4/CD8 ratio of ten or more, 41 patients had evidence of a chromosomally abnormal clone in the blood, and 23 patients had relative or absolute lymphocytosis with molecular evidence of a dominant T cell clone. Altogether one criterion was present in nine patients, two criteria were present in 11 patients, three criteria were present in 12 patients, and all four criteria were present in 16 patients. The only patient without any of these criteria was a patient with SS who had been heavily pretreated with suppression of blood lymphocyte counts, but with 45% Sézary cells per 100 lymphocytes on smears. Two patients had a CD4/CD8 ratio exceeding 10, but without an increase in CD4+ cells: one patient had SS with coexisting B-cell chronic lymphocytic leukemia, the other patient only had a depressed CD8 count. In addition, many of the cases had evidence of phenotypically abnormal T cells by flow cytometry, and an increased percentage of CD4+CD7- or CD4+CD26- subsets in the lymphocyte gate of 40% and 30% or more (ISCL tentative criteria for leukemic involvement) (Bernengo *et al*, 2001; Vonderheid *et al*, 2002; Vonderheid and Bernengo, 2003), was found in 23 and 34 patients, respectively.

Literature review The literature was reviewed for cases of leukemic CTCL in which the neoplastic cells in the blood were characterized for V β expression using various methods (Table S2). An effort was made to avoid counting cases more than once. A total of

136 cases were identified (Table S2; Fig S1). V β 5 usage was identified in 30 (22%) of the cases followed by V β 8 and V β 13 (each 11%). When V β 5 expression was further characterized into sub-families, 13 of 26 (50%) cases that were studied expressed V β 5.1. Of interest, bclonal expression of V β 1/V β 14 and V β 10/V β 18 was observed in two cases of SS studied by Bahler (not counted in the review) (Bahler *et al*, 1992).

Studies supported by the Leonard and Ruth Levine Skin Research Fund.

Supplementary Material

The following material is available from <http://www.blackwellpublishing.com/products/journals/suppmat/JID/JID23586/JID23586sm.htm>

Figure S1

V β 5 expression in leukemic CTCL is increased relative to other V β segments.

Figure S2

The percentage of V β + neoplastic cells correlates significantly but weakly with the percentage of CD4+CD7- (panel A) but not CD4+CD26- (panel B) in this series.

Table S1. Patients with leukemic cutaneous T cell lymphoma studied with anti-V β antibodies.

Table S2. V β family usage in cutaneous T cell lymphoma, leukemic phase

Supplementary references

DOI: 10.1111/j.0022-202X.2004.23586.x

Manuscript received June 20, 2004; revised August 15, 2004; accepted for publication September 20, 2004

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