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Most $CD8^+$ Cells in Skin Lesions of $CD3^+CD4^+$ Mycosis Fungoides Are $CD3^+$ T Cells That Lack CD11b, CD16, CD56, CD57, and Human Hanukah Factor mRNA

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To define further the characteristics of $CD8^+$ cells in skin lesions of $CD3^+CD4^+$ mycosis fungoides (MF), the authors used single- and double-label immunohistologic techniques and* in situ *hybridization to detect antigens and transcripts associated with certain types of cytotoxic or suppressor function. The cytotoxic markers included CD16, CD56, CD57, and an anti-sense probe for human Hanukah factor (HuHf) mRNA. Analysis of 23 cases demonstrated that lesional $CD8^+$ cells were $CD3^+$ T cells that generally lacked expression of any of the cytotoxic markers studied. Analysis of another 10 cases confirmed the $CD3^+$ T-cell lineage of lesional $CD8^+$ cells and demonstrated that these cells also lacked expression of the suppressor-associated marker, CD11b. In aggregate, these results indicate that most $CD8^+$ cells in $CD3^+CD4^+$ MF skin lesions are of T-cell rather than NK-cell differentiation. Their overall phenotype suggests that they may be major histocompatibility complex (MHC)-restricted cytotoxic T cells lacking appreciable levels of HuHF serine protease. Because the induction of $CD8^+$ suppressor T cells is mediated by $CD4^+$ T cells expressing the $CD45RA^+RO^-$ phenotype, CD45 epitope expression was studied in 15 MF cases. The vast majority (13/15) contained $CD3^+CD4^+$ tumor cells that were $CD45^+RA^-RB^+RO^+2B11^+$. This phenotype is consistent with memory T cells rather than suppressor-inducer T cells, and correlates with the paucity of phenotypically defined suppressor T cells in $CD3^+CD4^+$ MF skin lesions. (Am J Pathol 1991, 138:1545–1552)

The vast majority of cases of mycosis fungoides (MF) are lymphomas of $CD3^+CD4^+$ T cells.[1] Almost all MF skin lesions contain a subpopulation of $CD8^+$ cells admixed with these tumor cells, yet the role of these cells in the pathophysiology of MF remains unclear. Although rare $CD8^+$ helper T cells have been reported, $CD8^+$ cells generally are confined to one or more of the following subsets: major histocompatibility complex (MHC)-restricted cytotoxic T cells, non–MHC-restricted cytotoxic T cells, suppressor T cells, $CD8^+$ precursor T cells, or natural killer (NK) cells.[2] These $CD8^+$ subsets express characteristic patterns of antigens and mRNA that correlate well with their functional capabilities *in vitro*.[2–6] These correlations are summarized in Table 1.

In the current study, we have used *in situ* hybridization, two-color immunofluorescence, and single-label immunoperoxidase methods to characterize $CD8^+$ cells in $CD3^+CD4^+$ MF skin lesions. Our findings suggest that most of these $CD8^+$ cells are probably of cytotoxic T-cell lineage.

Methods

Patients

Skin biopsy specimens were obtained from the Cutaneous Lymphoma clinics at Stanford University and Case

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Table 1. *Possible Subsets of Functionally Mature CD8$^+$ Lymphoid Cells*

Cell Subset*	CD3	CD57	CD11b	CD16	CD56	CD28	S100	HuHFmRNA
Cytotoxic T cell (MHC-restricted)	+	−	−	−	−	+	−	+/−
Cytotoxic T cell (Non–MHC-restricted)	+	+/−	(+)	−	+	+	−	+
Suppressor T cell	+	−	+	−	−	−	+/−	−
Natural killer cell	−	+/−	(+)	+	+	+	−	+

+ Vast majority of cells express the marker.
(+) Cells express low levels of the marker.
− No/few cells express the marker.
* CD8$^+$ helper T cells have been described in functional studies of T-cell clones, but these are believed to be at most a rare component of lesional T-cell infiltrates.[2]

Western Reserve University. Sixty-six skin biopsies obtained from 43 patients were included in this study. All biopsies showed histopathologic features diagnostic of mycosis fungoides[1] except three biopsies from three patients with large plaque parapsoriasis and two biopsies from two patients with non-MF cutaneous T-cell lymphoma. All MF biopsies were studied with an extensive panel of monoclonal antibodies (MAbs)to establish the phenotype of the tumor cells, which was CD3$^+$CD4$^+$ in all cases except one, which was CD3$^+$CD8$^+$. Cytotoxic-associated markers (CD16, CD56, CD57, HuHF mRNA) were assessed in 23 biopsies from 23 MF patients (see Results). A suppressor-associated marker (CD11b) was assessed in 10 biopsies from 10 MF patients (see Results). CD45 (leukocyte common antigen) epitopes were assessed in 38 biopsies from 15 MF patients, and one biopsy from each of the patients with large plaque parapsoriasis and non-MF cutaneous T-cell lymphoma (see Results).

Antibodies

The MAbs used in various portions of this study are listed in Table 2.

The markers associated with cytotoxic function that were assessed included three antigens (CD16, CD56, and CD57) and human Hanukah factor (HuHF) mRNA. The first three antigens are expressed by NK cells and the subset of cytotoxic T lymphocytes (CTLs) that are non-MHC restricted. Human Hanukah factor mRNA and its murine counterpart, HF mRNA, encode a serine protease (granzyme A) associated with NK cells and CTLs in general. Murine allografts undergoing rejection contain HF mRNA$^+$ cells that are almost exclusively CD4$^-$CD8$^+$.[2] Another marker expressed by CD8$^+$ cytotoxic T cells is CD28.[3] Our prior studies of T cells in acquired immune deficiency syndrome (AIDS)-related diseases indicated that CD28 expression is often weak and difficult to assess even with the signal amplification attained with multistage immunostaining methods.[7,8] Therefore this marker was excluded from our studies.

The markers associated with suppressor T-cell function that could potentially be assessed included two antigens (CD11b and S100 β). Suppressor T cells are restricted to the CD8$^+$CD11b$^+$CD28$^-$ subset.[4,5] Approximately one half of this subset is also S100 β$^+$ (Table 1). Preliminary trials of anti-S100 MAb S161 showed the expected staining of Langerhans cells and nerve fibers but also a peculiar punctate staining of most cells in MF skin lesions, including keratinocytes. This marker was therefore not included in our study.

Single-label Immunoperoxidase

Immunoperoxidase analysis was performed using a three-stage method (MAb/biotinylated goat anti-mouse gamma G immunoglobulin [IgG]/avidin-horseradish peroxidase) as described previously.[9]

Double-label Immunofluorescence

Two-color immunohistologic analysis was performed using the following five-stage method: MAb A/F(ab')$_2$ frag-

Table 2. *Monoclonal Antibodies and an RNA Probe Used to Characterize CD8$^+$ Cells*

T-Cell Markers (CD Designation)
Anti-Leu2 (CD8)*: Cytotoxic/Suppressor T cell
Anti-Leu3 (CD4)*: Helper/Inducer T cell
Anti-Leu4 (CD3)*: Pan T cell
Cytotoxic Markers (CD Designation)
Anti-Leu7 (CD57)*
Anti-Leu11b (CD16)*
Anti-Leu19 (CD56)*
HuHF antisense RNA†
Suppressor Marker (CD Designation)
Anti-Leu15 (CD11b)*
Leukocyte Common Antigen Markers (CD Designation)
2D1 (CD45)*
T29/33 (CD45)‡
2B11 (CD45)§
Anti-Leu18 (CD45RA)*
PD7/26 (CD45RB)§
UCHL1 (CD45RO)‖

Markers were obtained from the following sources: Becton Dickinson, Mountain View, CA*; Irving Weissman, Stanford University†; Boehringer Mannheim, Indianapolis, IN‡; David Mason, Oxford University§; Dako, Carpenteria, CA.‖

ments of goat anti-mouse IgG conjugated to tetramethylrhodamine isothiocyanate (TRITC)/irrelevant blocking MAb of the same isotype as MAb B/ biotinylated MAb B/ avidin conjugated to Fluorescein isothiocyanate (FITC). Controls included deletion of individual stages and substitution of isotype-matched antibodies of irrelevant specificity.

In Situ *Hybridization*

In situ hybridization was performed as described previously using an anti-sense RNA probe for HuHF mRNA.[2] A HuHF RNA sense probe served as the negative control, while a cross-reactive murine cytotoxic cell clone (3A2) served as a positive control.

Results

Characterization of Cytotoxic Markers in MF

We used both immunoperoxidase and *in situ* hybridization techniques to detect cells expressing various cytotoxic phenotypes within the lesional skin of 23 patients with $CD3^+CD4^+$ MF. These included nine patients with nonatrophic patch/plaque lesions, and seven patients each with erythroderma or poikiloderma.

The median percentage of $CD8^+$ cells in our cases was 20, with a range of less than 1% to 50%. The median percentage of cells expressing the greatest value of any of the cytotoxic-associated markers was less than 1%, with a range of 0% to 10% (Figures 1 and 2). By comparing these data on a case-by-case basis, we estimated the maximum possible percentage of $CD8^+$ cells that expressed cytotoxic-associated markers or were of non–T-cell lineage. The median percentage of such cells was 2%, with a range of 0% to 100%. No significant differences in any of these values were noted among the various types of skin lesions on which biopsies were performed.

Another MF patient (one of the 10 discussed below) had focal areas containing both $CD8^+$ cells and $CD56^+$ cells. To determine if these antigens were co-expressed, two-color immunofluorescence was performed and demonstrated two distinct subpopulations: $CD8^+$ $CD56^-$ and $CD8^-CD56^+$. $CD8^+CD56^+$ cells were not identified. Additional two-color studies confirmed the impression, based on examination of immunoperoxidase-stained serial sections, that virtually all $CD8^+$ cells were $CD3^+$. In aggregate, these findings suggested that most $CD8^+$ cells infiltrating the skin lesions of $CD3^+$ $CD4^+$ MF are T cells that lack detectable CD16, CD56, CD57, and HuHF mRNA.

Characterization of Suppressor Markers in MF

We used immunoperoxidase and two-color immunofluorescence techniques to detect cells expressing a $CD8^+CD11b^+$ suppressor T-cell phenotype within the

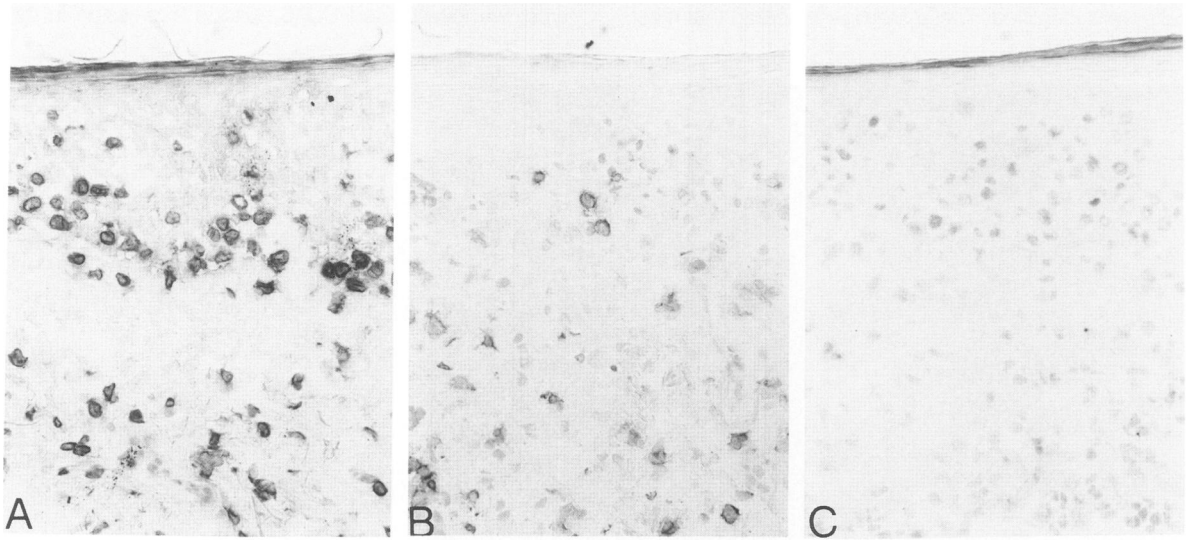

Figure 1. *Most $CD8^+$ cells in MF skin lesions are $CD3^+$ but lack markers associated with certain types of cytotoxic function.* **A:** *Immunoperoxidase-stained cryostat section containing an epidermotropic, interface infiltrate of $CD3^+$ lymphocytes.* **B:** *Semiserial section showing that a subset of cells are $CD8^+$.* **C:** *Semiserial section showing that none of these cells are $CD16^+$. In general, very few cells were $CD16^+$, $CD56^+$, or $CD57^+$ (methylene blue counterstain, ×800).*

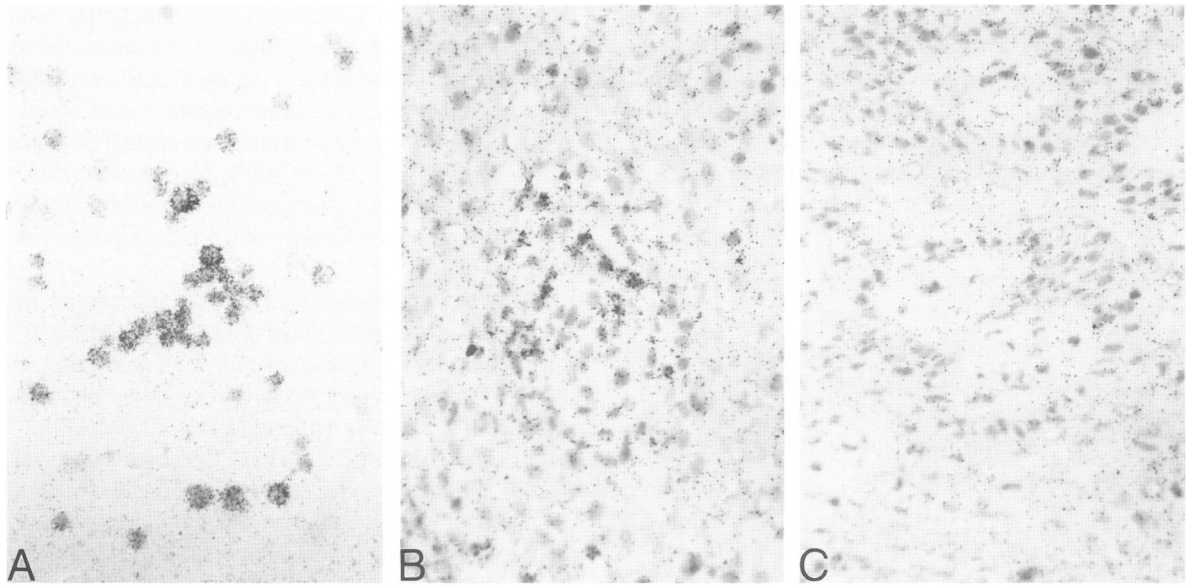

Figure 2. *Cells containing HuHF transcripts are rare in MF skin lesions.* **A:** *3A2 cell line (positive control) showing dense silver grains overlying cells containing HuHF mRNA as detected by* in situ *hybridization using an anti-sense RNA probe followed by autoradiography.* **B:** *A small focus of mononuclear cells containing HuHF mRNA in the upper dermis of a MF skin lesion cryostat section. In general, these cells were rare.* **C:** *Semiserial section with* in situ *hybridization performed using a HuHF RNA sense probe (negative control). Only a diffuse, low-level background signal is present (neutral red counterstain. ×800).*

lesional skin of 10 patients with $CD3^+CD4^+$ MF. All 10 patients had patch/plaque lesions.

In immunoperoxidase-stained sections, there was a variable minority of $CD11b^+$ cells in each lesional biopsy. These cells generally had the morphologic features of macrophages or granulocytes; however some were similar in size and shape to $CD8^+$ cells. We therefore performed two-color immunofluorescence studies on serial sections of these same biopsies (Figure 3). Most of the $CD8^+$ cells were $CD11b^-$. Only rare $CD8^+CD11b^+$ cells were identified. These findings indicated that most $CD8^+$ cells infiltrating $CD3^+CD4^+$ MF skin lesions lack detectable features associated with suppressor T-cell function.

Characterization of CD45 Antigen Expression in MF

Because the induction of suppressor T cells is mediated by $CD4^+$ T cells expressing the $CD45RA^+RO^-$ phenotype, we investigated CD45 epitope expression in the

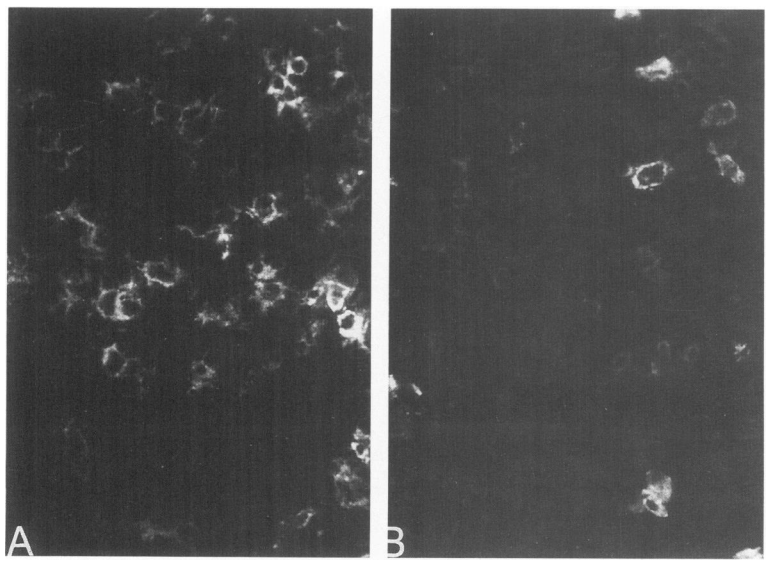

Figure 3. *Most $CD8^+$ cells in MF skin lesions lack a marker associated with suppressor function.* **A:** *Two-color immunofluorescence-stained cryostat section showing location of $CD11b^+$ cells (red, $TRITC^+$).* **B:** *Same field of same section showing location of $CD8^+$ cells (green, $FITC^+$). No $CD8^+CD11b^+$ cells are identified. In general, $CD8^+CD11b^+$ cells were very rare (×800).*

same 10 patients studied for CD11b expression plus five others who also had patch/plaque lesions. A total of 30 biopsies (three to five each) had been obtained from seven of these patients; therefore the total number of biopsies studied for CD45 expression was 38.

Tumor cells were recognized as CD4$^+$ T cells within Pautrier microaggregates and smaller intraepidermal cell groups, except in one case in which tumor cells were CD8$^+$. In most cases (13/15), tumor cells expressed the CD45 phenotype associated with memory T cells (CD45RA$^-$RO$^+$). Their complete CD45 phenotype was CD45$^+$RA$^-$RB$^+$RO$^+$2B11$^+$. In one case, the tumor cells were CD45$^+$RA$^+$RB$^+$RO$^-$2B11$^+$. In the one case of CD8$^+$ MF, tumor cells were CD45$^+$RA$^+$RB$^+$RO$^+$ 2B11$^+$. The pattern of tumor cell CD45 epitope expression was consistent in all serial biopsies. Most CD4$^+$ lesional T cells in three cases of large plaque parapsoriasis and two cases of CD4$^+$ non-MF cutaneous T-cell lymphoma were also CD45$^+$RA$^-$RB$^+$RO$^+$2B11$^+$.

A variable minority of CD45RA$^+$ cells were present in each biopsy of CD45RA$^-$ MF. Based on their morphology and distribution relative to CD3$^+$ cells in serial sections, these cells were generally regarded as T cells. To determine whether these CD45RA$^+$ T cells were CD4$^+$ or CD8$^+$, two-color immunofluorescence was performed for CD8 and CD45RA antigens in one biopsy from each of the 10 cases studied earlier for CD11b expression. The results indicated that most CD8$^+$ cells were CD45RA$^-$. Only approximately 10% to 20% of CD8$^+$ cells were CD45RA$^+$ (Figure 4). Based on the low percentage of CD8$^+$CD45RA$^+$ host T cells, we would expect that at least some of the CD4$^+$ host T cells in typical MF lesions coexpress CD45RA; however an accurate percentage could not be derived from our data because attempts at two-color immunofluorescence for CD4 and CD45RA antigens were technically unsatisfactory. This was due to the relatively weak labeling achievable with the biotinylated anti-CD4 antibody used in our two-color immunostaining system.

Discussion

The results of this study of 33 MF patients establish the following cellular marker profile for the vast majority of CD8$^+$ cells in lesional skin biopsies: CD3$^+$CD11b$^-$ CD16$^-$CD56$^-$CD57$^-$HuHFmRNA$^-$. The expression of CD3 strongly suggests that these cells are T cells rather than NK cells. The absence of CD11b, CD16, CD56, and CD57 also suggests that these lesional CD8$^+$ cells are not NK cells. Furthermore it suggests that they are neither suppressor T cells nor non–MHC-restricted cytotoxic T cells (Table 1). The lack of detectable HuHF mRNA suggests that these CD8$^+$ cells do not express appreciable levels of HuHF (granzyme A), which is a granule-associated serine protease. Such proteases are found in many but not all types of cytotoxic cells.[10] In aggregate, the marker profile is most consistent with the interpretation that the lesional CD8$^+$ cells are probably of MHC-restricted cytotoxic T-cell lineage but lack significant levels of HuHF. It remains to be determined whether the lack of detectable HuHF is due to functional immaturity of the CD8$^+$ cells or related to expression of alternative cytotoxic-associated factors such as perforin (a pore-forming protein reminiscent of the complement membrane attack complex) or TIA-1 (a novel 15-kd granule-associated protein expressed preferentially by cytotoxic cells).[10] A less likely possibility is that functiuonally significant levels of

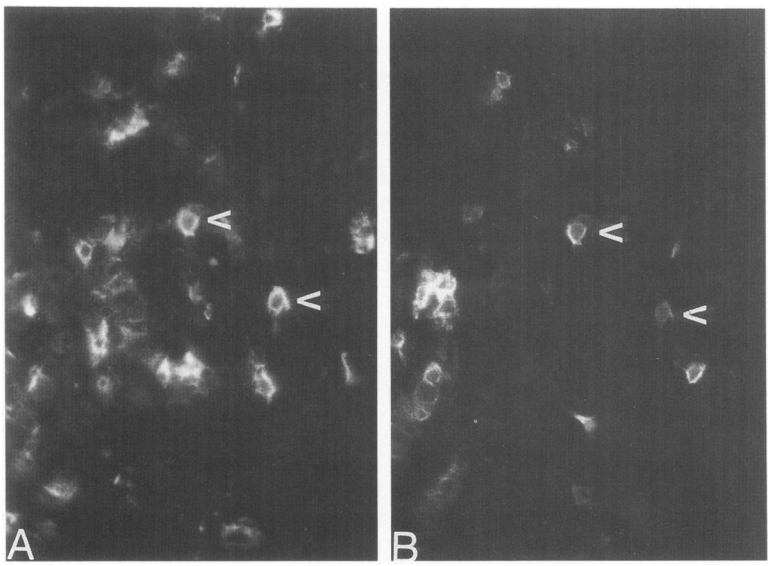

Figure 4. *A minority of CD8$^+$ cells in MF skin lesions are CD45RA$^+$.* **A:** *Two-color immunofluorescence-stained cryostat section showing location of CD45RA$^+$ cells (red, TRITC$^+$).* **B:** *Same field of same section showing location of CD8$^+$ cells (green, FITC$^+$). A small number of CD8$^+$CD45RA$^+$ cells are identified (arrowheads). In general, approximately 10% to 20% of CD8$^+$ cells were also CD45RA$^+$ (×800).*

HuHF mRNA are present in these cells but that the amount is too small to be detectable with the techniques used in this study.

Whether the $CD8^+$ T cells in $CD3^+CD4^+$ MF are functionally mature effector cells, it is unknown if they have been specifically recruited to MF lesions or if they are 'bystander' cells nonspecifically recruited to the lesional skin infiltrate by cytokines or other factors. This possibility might help explain why the number of lesional $CD8^+$ cells showed no correlation with prognosis in one large series of MF patients.[11] In this context, it is important to note that experimental models of cell-mediated immune reactions have shown that most lesional T cells may be nonspecifically recruited because cell-mediated immune reactions can be induced by a very small number of specifically sensitized effector cells.[2]

There is relatively little literature available concerning similar studies of $CD8^+$ cell subsets in skin diseases other than MF. One study of human graft-*versus*-host disease, however, found that the $CD8^+$ cells in lesional infiltrates were generally $CD11b^-$, supporting the interpretation that they were probably $CD8^+$ cytotoxic T cells.[12] This conclusion is supported by studies of murine allograft rejection and experimental lymphocytic choriomeningitis in which *in situ* hybridization and immunohistologic analysis showed a good correlation between $CD8^+$ T cells and HF $mRNA^+$ cells.[13,14] Furthermore the maximal number of HF $mRNA^+$ cells correlated well with the maximal cytotoxic functional activity of the infiltrating cells.[14] In contrast, preliminary studies of a small number of skin biopsies from patients with psoriasis, lichen planus, and acute allergic contact dermatitis demonstrated that only occasional $CD8^+$ T cells expressed certain cytotoxic-associated antigens or mRNA.[2]

It will be important to expand this database in human skin diseases to include a detailed analysis of $CD8^+$ cells for cytotoxic and suppressor markers. We would anticipate, however, that each disease will have its own unique characteristics, making comparisons between two different diseases a problem of 'apples and oranges.' Furthermore there might not necessarily be any simple relationship between $CD8^+$ cell subsets and features such as disease chronicity, even within a single disease spectrum. For example, the principal difference between acute, self-limited and chronic, persistent allergic contact dermatitis in many cases might be disappearance *versus* persistence of the exogenous antigenic stimulus rather than the effects of lesional cytotoxic or suppressor $CD8^+$ T cells. Similarly the spontaneously resolving yet recurrent crops of lesions characteristic of pityriasis lichenoides et varioliformis acuta and lymphomatoid papulosis might depend on an influx of $CD8^+$ suppressor T cells capable of down-regulating the entire lesional infiltrate, or might instead depend on other factors such as $CD8^+$ cytotoxic T-cell–mediated destruction of epidermal cells bearing some target antigen, or perhaps an inherent cyclic variation in the proliferative activity of the clonal T cells often detectable in these lesions by Southern blot analysis.[15,16]

The CD45 epitope mapping studies in 38 lesional skin biopsies from 15 MF patients indicated that the tumor cells in most cases express the $CD45RA^-CD45RO^+$ phenotype associated with memory T cells. The tumor cells in most cases lack the $CD45RA^+CD45RO^-$ phenotype associated with naive T cells, which include the $CD4^+$ T-suppressor–inducer population that mediates suppressor T-cell differentiation.[17] Thus if $CD4^+$ MF tumor cells have retained any functional activity, it would not be expected to include the ability to activate suppressor T cells. This is consistent with the paucity of phenotypically identifiable suppressor T cells among the lesional $CD8^+$ cells in this study. Although studies comparing the number of phenotypically defined $CD4^+$ suppressor-inducer T cells with the number of phenotypically defined $CD8^+$ suppressor T cells remain to be completed for diseases other than MF, the lack of expression of the $CD45RA^+CD45RO^-$ suppressor-inducer phenotype by MF tumor cells has been noted in at least one other study.[18] Furthermore the suppressor-inducer T-cell population also has been noted to be decreased in the peripheral blood of MF patients.[19]

Our studies do however provide indirect evidence that MF cases may contain some non-neoplastic $CD4^+CD45RA^+$ T cells. In fact, such cells were cloned from lesional skin in one case of MF and were shown to exhibit functional capabilities consistent with T-suppressor–inducer cells *in vitro*.[20] It is possible that such cells do not induce suppressor cells effectively in MF lesions *in vivo* either because they are too few in number or because they are inhibited by various factors. The induction of suppressor T cells is believed to require cell-surface contact between the $CD8^+$ suppressor T-cell precursor and the $CD4^+CD45RA^+$ suppressor-inducer T cell.[3] If suppressor T-cell precursors contact only $CD4^+CD45\text{-}RA^-$ MF tumor cells rather than $CD4^+CD45RA^+$ suppressor-inducer T cells, then they would not be expected to differentiate into functionally mature effector cells. Similarly, $CD4^+$ T cells are also involved in the induction of functionally mature $CD8^+$ cytotoxic T cells from their precursors.[21] If $CD4^+$ MF tumor cells are the principal $CD4^+$ T-cell subset available for such an induction, and if they are functionally incapable of serving this role, this might result in a paucity of mature $CD8^+$ cytotoxic T cells in MF lesions.

The complete CD45 profile of MF has not been reported previously. Our data demonstrate three subsets of disease. In most cases, the tumor cells are $CD45^+RA^-RB^+RO^+2B11^+$, whereas in some cases,

Table 3. *Relationship Between CD45 Isoform and CD45 Epitope Expression*

CD45 isoform (MW)	CD45 epitope designation			
	CD45	CD45RA	CD45RB	CD45RO
220 kd	+	+	+	−
205 kd	+	+	+	−
205 kd	+	−	+	−
190 kd	+	−	+	−
180 kd	+	−	−	+

MW, molecular weight.

the tumor cells are $CD45^+RA^+RB^+RO^-2B11^+$ or $CD45^+RA^+RB^+RO^+2B11^+$. Each CD45 epitope is known to be restricted to one or more of the five known isoforms of the CD45 molecule (Table 3).[22] Based on our epitope mapping, it is apparent that in most cases of MF, the tumor cells probably express at least two, and possibly three, CD45 isoforms but lack expression of the two highest molecular-weight isoforms. In contrast, MF tumors expressing the less common CD45 epitope patterns probably express at least one of the two highest molecular-weight CD45 isoforms.

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