

Leukemic T Cells from Patients with Cutaneous T-Cell Lymphoma Demonstrate Enhanced Activation Through CDw60, CD2, and CD28 Relative to Activation Through the T-Cell Antigen Receptor Complex

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Antigen-dependent activation of T cells occurs through the T-cell antigen-receptor complex (TCR/CD3). Antigen-independent T-cell activation may occur through the surface molecules CDw60, CD2, and CD28. We wished to determine whether these antigen-independent T-cell-activation pathways could be involved in proliferation of leukemic T cells from patients with cutaneous T-cell lymphoma (CTCL). Whereas CDw60 was only expressed on $28\% \pm 7\%$ (mean $\pm$ SEM) of blood T cells obtained from healthy control subjects ($n = 4$), CDw60 was expressed on $94\% \pm 3\%$ of blood T cells obtained from patients with CTCL ($n = 4$). Dual color immunofluorescence microscopy of the T-cell infiltrate in involved skin of these patients demonstrated that almost 100% of the T cells expressed CDw60. Not only did T cells in the patients with CTCL express CDw60, but triggering of the T cells with anti-CDw60 resulted in enhanced

proliferation relative to anti-TCR/CD3 and mitogenic lectins. Other antigen-independent pathways also appeared highly active in the T cells from patients with CTCL because enhanced proliferation relative to anti-TCR/CD3 or mitogenic lectins was found when anti-CD2 or anti-CD28 plus phorbol ester was used as stimulant. Despite the brisk proliferation induced by anti-CDw60, anti-CD2, or anti-CD28, T cells from the patients did not produce detectable amounts of γ -interferon. The inability to produce γ -interferon correlates with our finding of absent ($n = 3$) or weak ($n = 1$) intercellular adhesion molecule-1 expression in the lesional keratinocytes in these patients. In conclusion, T cells of patients with CTCL demonstrate elevated expression of a T-cell-independent signaling molecule CDw60 and respond to antigen-independent activating signals. *J Invest Dermatol* 100:667-673, 1993

Cutaneous T-cell lymphomas (CTCL) are neoplasms of T cells that primarily affect the skin [1]. Sezary syndrome is a leukemic variant of CTCL characterized by erythroderma, pruritus, lymphadenopathy, and circulating atypical, presumably malignant T cells with cerebriform nuclei [2]. The skin lesions in CTCL contain a polymorphic cell infiltrate comprised of both malignant T cells and non-malignant tumor-infiltrating T lymphocytes (TIL), as well as macrophages, Langerhans cells, and other inflammatory cells [1,3].

Whereas CTCL TIL may be responsive to antigen-receptor-dependent signaling provided by hyperstimulatory antigen-presenting Langerhans cells present in involved epidermis [4], the malignant clone may proliferate and achieve its activated state through alternative antigen-independent activation pathways. Antigen-independent pathways that might be involved in activation of the malignant T cells include ligand triggering of molecules, such as CDw60 [5], CD2 [6], and CD28 [7]. Ligands for both CD2 and CD28 have been identified as lymphocyte function-associated antigen (LFA)-3 and B7/BB-1, respectively [8,9]. LFA-3 is expressed

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Abbreviations:

Con A: Concanavalin

CTCL: cutaneous T-cell lymphoma(s)

ICAM-1: intercellular adhesion molecule-1

HLA: human leukocyte antigen

Ig: immunoglobulin

IL-2: interleukin-2

LFA: lymphocyte function-associated antigen-1

MNC: mononuclear cell(s)

PHA: phytohemagglutinin

PMA: phorbol myristate acetate

TCR: T-cell receptor

[3H]thymidine: TdR

TIL: tumor-infiltrating T lymphocytes

Table I. Clinical Characteristics of Four Male Patients with CTCL

Patient	Age (years)	Total Leucocyte Count ^a	Clinical Status ^b	Bone Marrow Involvement ^c	TNM	TCR Rearrangement ^d
1	71	50.0	3, 4	+	T ₁ N ₀ M ₀ B ₁	+
2	55	16.0	1, 3-9	ND	T ₄ N ₃ M ₀ B ₁	+
3	65	23.0	1, 3-6, 9	+	T ₄ N ₃ M ₀ B ₁	+
4	72	11.0	1-4, 6, 8, 9	-	T ₄ N ₀ M ₀ B ₁	+

^a Normal leucocyte range $3.0-9.0 \times 10^9/l$.

^b 1, erythroderma; 2, hepatomegaly; 3, skin plaque or tumor; 4, intense pruritus; 5, adenopathy; 6, alopecia; 7, onychodystrophy; 8, keratoderma of palms and soles; 9, ectropion with resultant ocular inflammation.

^c (+) Present; (-) not present; ND, not done.

^d Presence of T-cell receptor (TCR) β gene rearrangement (non-germ-line bands) in both the skin and peripheral blood; (+) present.

on both keratinocytes and Langerhans cells [10], as may be B7.†† The ligand for CDw60 has not yet been identified. The responses of leukemic blood T cells from patients with CTCL to various T-cell antigen-independent stimuli were therefore compared with antigen-dependent stimuli.

MATERIALS AND METHODS

Patients Four untreated patients with clinical, laboratory, and histologic features of CTCL and four healthy adult control subjects participated in the study (Table I). Each patient had evidence of blood involvement by peripheral smear and Southern blot analysis. Patient 1 was characterized as having leukemic CTCL and patients 2-4 as having Sezary syndrome.

Immunohistochemistry Skin biopsies were taken from involved skin of four patients with CTCL and were immediately snap-frozen and stored in liquid nitrogen. Immunohistologic staining was performed on cryostat sections using a three-stage biotin-avidin horseradish peroxidase procedure, as previously described [11]. A panel of antibodies was applied to each biopsy (Table II). Anti-CDw60 was diluted 1:200 and anti-CD28 1:50; all other antibodies were diluted 1:10. The specificity of the monoclonal antibodies used in the staining procedures was verified by use of irrelevant isotype antibodies, GFAP and Gp IIb/IIIa (Table II). Sections of tonsil and lymph nodes were used as positive controls for the antibodies. The slides were counterstained with hematoxylin/eosin and scored in a blinded fashion. In each section, 150 cells of the lymphohistiocytic infiltrate were analyzed; the number of cells expressing the respective antigen is given in percent of infiltrating cells.

Immunofluorescence Staining Blood mononuclear cells (MNC) were stained by a two-stage procedure [4]. The primary antibodies were used in a 1:10 dilution. As secondary antibody, rhodamine-conjugated goat anti-mouse immunoglobulin (Ig) at a dilution of 1:100 (Dakopatts, Copenhagen, Denmark) was used. Double color immunofluorescence microscopy of T cells in the skin was performed by using rhodamine-conjugated goat anti-mouse IgM (Tago Inc., Burlingame, CA) to visualize anti-CDw60 (IgM antibody), and fluorescein-conjugated goat anti-mouse IgG (Kirkegaard, Gaithersburg, VA) to visualize the co-staining anti-CD3. Peripheral blood T cells were double-stained using rhodamine-conjugated goat anti-mouse IgM (Serotec, Oxford, England) and fluorescein-conjugated goat anti-mouse IgG1 (Serotec). To control for cross-reactivity and non-specific binding of the second antibodies, irrelevant primary isotype antibodies GFAP and Gp IIb/IIIa were substituted for anti-CD3 or anti-CDw60. The sections were evaluated by an immunofluorescence microscope equipped with epi-illumination and appropriate filter.

T-Cell Activation Fifty-thousand MNC were incubated with 1 μ g/ml phytohaemagglutinin (PHA) (Wellcome Diagnostics, Dartford, England), 10 μ g/ml Concanavalin A (Con A) (Calbiochem Corp., San Diego, CA), pokeweed mitogen (1:200) Gibco, Grand island, NY), anti-CDw60 (1:200), a mitogenic pair of anti-CD2 (1mono2A6 and 10LD24C1, each at 1:500), anti-CD2 (1:250), anti-CD28 (1:200), anti-T-cell receptor (TCR)-1 (1:250), anti-CD3 (1:500), or 50-U recombinant interleukin-2 (IL-2) (Amersham, Arlington Heights, IL), with or without phorbol myristate acetate (PMA) (Sigma, St. Louis, MO) at 3 ng/ml in 200 μ l RPMI 1640 with 10% AB serum, glutamine, and antibiotics. The cultures were pulsed after 48, 72, or 96 h with 1 μ Ci of [3H]thymidine (TdR)/well and were harvested 18 h later on a cell harvester (Teknunc, Roskilde, Denmark); TdR incorporation was measured with a liquid scintillation counter.

Determination of γ -Interferon The supernatants were tested for production of γ -interferon after 48 h of incubation with mitogenic lectins or antibodies, as mentioned above, with commercial enzyme-linked immunosorbent assay kits (γ -interferon; Holland Biotechnology, Leiden, The Netherlands).

Table II. Monoclonal Antibodies

Antibody	Specificity	Source ^a
CD4	T-helper cells	BD/DAKO
CD8	T-suppressor/cytotoxic cells	BD/DAKO
CD3	Pan T cell	BD/DAKO
CD2	Pan T cell (Erythrocyte rosette receptor)	BD/DAKO
T11 ₂ (1mono2A6)	Pan T cell (Erythrocyte rosette receptor)	^b
T11 ₃ (10LD24C1)	Pan T cell (Erythrocyte rosette receptor)	^b
HLA-DR	HLA-DR (class II antigens)	DAKO
Leu-M3	Monocytes/macrophages	BD
CD19	Pan B cells	DAKO
CD25 (anti-Tac)	IL-2 receptor	BD/DAKO
TCR	TCR antigen complex	BD
CDw60 (UM4D4)	A subset of T cells	OWN
CD28	80% of T cells	^c
CD45RO (UCHL1)	Higher expression on CD4 ⁺ helper/memory T cells	DAKO
CD45RA (2H4)	Higher expression on CD4 ⁺ suppressor/inducer T cells	CC
ICAM-1	Intercellular adhesion molecule-1	S
GFAP ^d	Acidic protein in brain and spinal cord	DAKO
Gp IIb/IIIa	Human platelets	DAKO

^a BD, Becton Dickinson Co., Mountain View, CA; CC, Coulter Clone, Hialeah, FL; DAKO, Dakopatts, Copenhagen, Denmark; S, Serotec, Oxford, England.

^b Kindly provided by Drs. Ellis Reinherz and Stuart Schlossman, Dana Farber Cancer Institute, Boston, MA.

^c Kindly provided by Dr. Laurence Turka, University of Michigan, Ann Arbor, MI.

^d Glial fibrillary acidic protein.

†† Fleming TE, Mirando WS, Elmetts CA: Expression of the activation antigen BB-1/B7 on cells of non-hematopoietic origin: keratinocytes from human epidermis (abstr). *J Invest Dermatol* 98:577A, 1992.

Table III. Immunophenotyping of the Lymphohistiocytic Dermal Infiltrate in Skin Biopsies from Patients with CTCL^a

	Patient Number			
	1	2	3	4
CD4	+++	+++	+++	++
CD8	—	+	+	++
CD2	+++	++	+++	+++
CD3	+++	+++	+++	+++
CD45RO	+++	+++	+++	+++
CD45RA	—	—	+	+
CD28	+++	++	++	++
CD19	—	—	—	—
CD25	—	—	+	—
CDw60	+++	+++	+++	+++
HLA-DR	++	++	++	ND
Leu-M3	—	—	—	—

^a (+) Sections with < 10% positive cells; (++) between 10% and 80%; (+++) > 80%; (—) negative for the respective antigen.

Skin-Derived T-Cell Clones Six-millimeter skin-punch biopsies were obtained from involved skin of a patient with tumor-stage mycosis fungoides-type CTCL and from a patient with CTCL. The biopsies were cut horizontally, as close to the epidermis as possible, and then cut into several 1-mm pieces and incubated in 24-well culture plates containing RPMI-1640 culture medium with 10% AB serum, 10% purified IL-2 (Electro-Nucleonics, Cell Science Laboratory, Silver Spring, MD), glutamine, and antibiotics at 37°C. Following 24 h of incubation, T cells were cloned using limiting dilution, as previously described [3].

T-Cell Lines from Peripheral Blood One-million MNC/ml obtained from a patient with CTCL and a healthy subject were stimulated with 1 µg/ml PHA (Wellcome) and 3 ng/ml PMA (Sigma) for 24 h. The cells were then extensively washed and cultured at a concentration of 1×10^6 cells/ml in RPMI-1640 culture medium with 10% AB serum, 10% purified IL-2 (Electro-Nucleonics), glutamine, and antibiotics at 37°C. The medium was changed twice weekly. When cell density was greater than 1×10^6 cells/ml, the cell cultures were split. After 1–3 weeks in culture, without repeated stimulation with PHA/PMA, analysis of CDw60 antigen was performed with a Becton Dickinson fluorescence-activated cell sorter IV flow cytometer. Anti-CDw60 was identified with fluorescein-conjugated goat anti-mouse Ig (Becton Dickinson). Analysis was performed on 10,000 cells, and the acquired data were expressed as percent of CDw60⁺ cells.

TCR Gene Rearrangement Southern blot analysis to detect rearrangement of the β -chain of the TCR was performed as previously described [3].

RESULTS

Lymphohistiocytic Infiltrate in Skin of Patients with CTCL Is Dominated by CD4⁺ CD45RO⁺ "Memory" T-Helper Cells That Also Express the CDw60 T-Cell-Activating Molecule To determine potential T-cell activation pathways in patients with CTCL, we examined activating molecules on T cells in the cellular infiltrate of the skin and in the peripheral blood. Table III depicts immunophenotyping of involved skin from the patients. In all specimens, CD3⁺ CD2⁺ T cells dominated. The dense bandlike dermal T-cell infiltrate was composed of CD4⁺ CD45RO⁺ "memory" T-helper cells. CD45RA⁺ T cells and CD8⁺ T cells were found only scattered within the infiltrate. A high percentage of the T cells were activated because they expressed the human leukocyte antigen (HLA)-DR class II antigen. Despite the activated state, only patient 3 demonstrated some expression of IL-2 receptor (CD25). CD19⁺ B cells and Leu-M3⁺ monocytes/macrophages were only occasionally seen.

Activated T cells normally produce γ -interferon, which upregu-

lates intercellular adhesion molecule (ICAM)-1; however, except for focal expression in patient 4, ICAM-1 (CD54) epidermal expression was absent. Occasional MNC in the dermis stained positive for ICAM-1 in patients 2 and 3 (data not shown).

Double immunofluorescence microscopy revealed that nearly 100% of the CD3⁺ T cells in lesional skin co-expressed the CDw60 molecule (Fig 1).

CDw60 Expression on Malignant T Cells In the peripheral blood from the patients with CTCL, the mean number of CDw60⁺ cells was $94\% \pm 3\%$ (mean $\pm$ SEM; $n = 4$) (Fig 1b; Table IV). In contrast, the expression of CDw60 on T cells in healthy control subjects was only $28\% \pm 7\%$ ($n = 4$).

Because nearly 100% of the lesional and peripheral blood T cells express CDw60, it is very likely that the malignant T cells express the molecule. To test this hypothesis, analysis of TCR gene rearrangements were performed to verify that a significant proportion of the cells comprised the malignant monoclonal T-cell population. DNA samples of both the blood and skin from patient 1 were digested with *Hind*III or *Eco*R1 and hybridized to a probe for the constant region of the TCR β to detect TCR β -chain gene rearrangements. As indicated on the Southern blot, the same rearranged bands both in the blood and the skin following *Hind*III digestion (Fig 2, arrows) and the near disappearance of the 12-kbp band both in the blood and skin following *Eco*R1 digestion demonstrate that the majority of the T cells in the blood and in the skin have the same TCR gene rearrangement and thus belong to the same clone.

Thus, CDw60 is expressed on malignant T cells because nearly 100% of the T cells express the molecule, and a majority of the T cells belong to a monoclonal population.

Non-Malignant Tumor-Infiltrating T Cells Derived from Patients with CTCL Demonstrate Normal Expression of CDw60 To demonstrate whether an enhanced expression of CDw60 also was present on tumor-infiltrating T cells, T cells obtained from the blood of patient 1 were cultured for 4 weeks in the presence of IL-2, PHA, and PMA. TCR gene rearrangement performed in the cells after 1–4 weeks in culture revealed that the malignant clone was gradually replaced by other clones, as indicated by the disappearance of the TCR gene rearrangement (Fig 3a). Staining with anti-CDw60 demonstrated that the level of expression on the T cells decreased simultaneously with replacement of the malignant clone with non-malignant TIL (Fig 3b). Thirteen tumor-infiltrating T-cell clones obtained from the skin of the same patient were also stained with anti-CDw60. The expression of CDw60 ($38\% \pm 8\%$, mean $\pm$ SEM) was comparable to that observed in normal blood T cells (Table IV) and in T-cell clones obtained from healthy peripheral blood [5]. In addition, 21 tumor-infiltrating T-cell clones obtained from a patient with mycosis fungoides in tumor stage demonstrated a mean of 25% CDw60⁺ T cells, despite a high level of CDw60 expression on the T cells in the tumor nodule (data not shown).

Anti-CDw60 as a Single Stimulus Potently Activates Blood T Cells from Patients with Leukemic CTCL Because patients with CTCL contained a high number of CDw60⁺ malignant T cells, we determined whether this molecule was involved in activation of the T cells. Proliferation in response to stimulation with anti-CDw60 was therefore compared with the response to various mitogenic lectins and T-cell surface molecules known to be involved in T-cell activation.

To determine the optimal time point for anti-CDw60 activation, a time study on the T cells from the patients with CTCL was performed. TdR incorporation reached a maximum after 48 h of culture, after which the incorporation decreased. In the following experiments, TdR incorporation was performed after 48 h of culture.

Consistent with the findings of others [12–17], patients with CTCL responded poorly or not at all to mitogenic plant lectins, including PHA, Con A, and PWM. Similar results were obtained

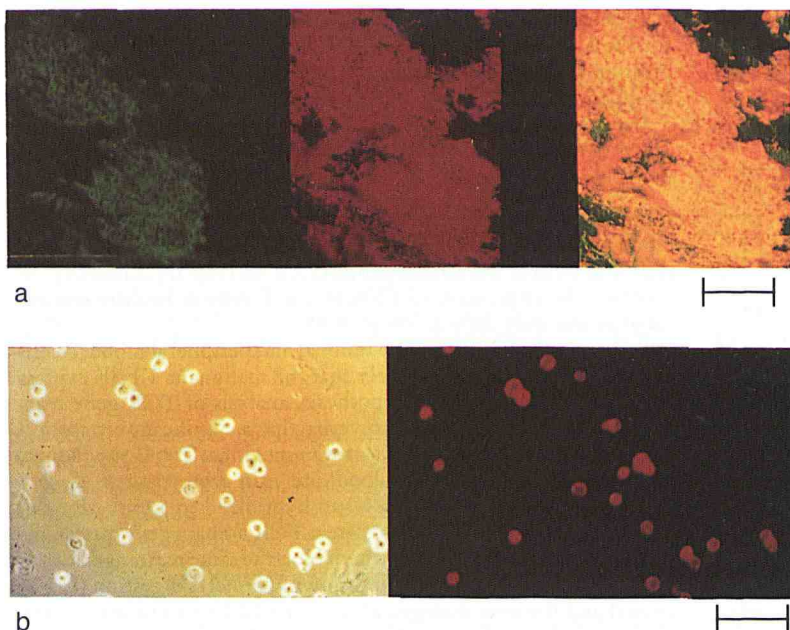


Figure 1. a) Double indirect immunofluorescence microscopy of a tumor nodule in the dermis from a patient with CTCL stained with anti-CD3 and anti-CDw60. CD3-positive cells are demonstrated by their green fluorescence. CDw60 cells are demonstrated by their red fluorescence. Double photographic exposure demonstrates that CD3⁺ and CDw60⁺ cells fluorescing both colors appear orange, demonstrating that nearly all the T cells in the skin co-express CDw60. Bar, 210 μ m. b) Indirect immunofluorescence of CDw60 on peripheral blood from a patient with CTCL. Left, phase microscopy; right, labeled by anti-CDw60 with rhodamine-conjugated goat anti-mouse IgM. Bar, 45 μ m.

using recombinant IL-2, anti-CD3, anti-TCR-1, anti-CD2, or anti-CD28 as single stimuli. In contrast, stimulation with anti-CDw60 resulted in a brisk proliferation in three of four patients (Fig 4, cross-hatched bars).

Because all of the above ligands, except anti-CDw60, activate T cells weakly when used as single stimuli, all of the mitogenic lectins and antibodies were co-cultured with PMA. For optimal activation through the CD2 pathway, a mitogenic pair of anti-CD2 antibodies were used.

In combination with PMA, all ligands induced activation of the T cells, but anti-CDw60 still demonstrated an enhanced response relative to the mitogenic lectins and anti-TCR/CD3 in all four patients studied. However, the mitogenic pair of anti-CD2 plus PMA and anti-CD28 plus PMA also demonstrated enhanced activation relative to mitogenic lectins and anti-TCR/CD3 (Fig 4, stippled bars).

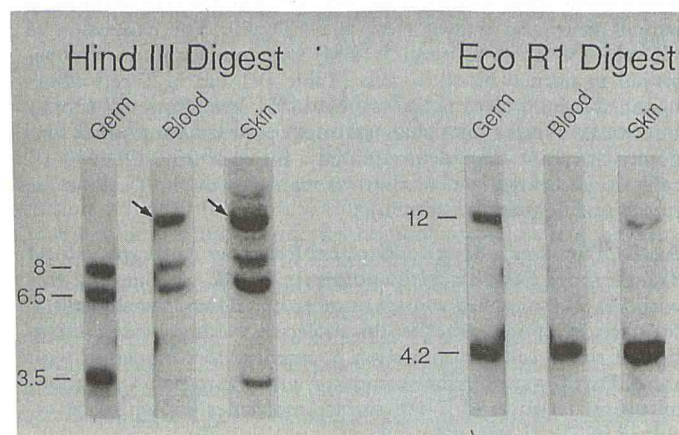


Figure 2. Southern blot analysis of a CTCL skin lesion and the peripheral blood from patient 1. DNA samples were digested with *Hind*III or *Eco*R1 and hybridized to the TCR- β probe to detect TCR β -chain gene rearrangements. Dashes, molecular sizes in kilobases of germ-line bands. As indicated on the Southern blot, the same rearranged bands both in the blood and the skin following *Hind*III digest (arrows) and the near disappearance of the 12-kbp band both in the blood and the skin following *Eco*R1 digest demonstrate that the majority of the T cells in the blood and in the skin have the same T-cell receptor gene rearrangement and thus belong to the same clone.

Antigen-Independent Pathways Dominate in Patients with CTCL Relative to MNC from healthy individuals, MNC from patients with CTCL reacted only weakly to stimulation by mitogenic lectins or anti-TCR/CD3. However, activation by anti-CDw60, anti-CD2, and anti-CD28 resulted in strong activation (Fig 5). Responses above the line indicate a more potent response from the patients with CTCL compared with healthy subjects. Thus, relative to T cells from healthy subjects, activation of T cells from patients with CTCL by antigen-independent pathways resulted in enhanced activation.

Capacity of Stimulated T Cells to Secrete γ -Interferon Is Significantly Diminished in T Cells from Patients with CTCL Compared with Healthy Subjects Certain differentiated T-cell subsets produce γ -interferon in response to activation (Th1), whereas others lack γ -interferon production (Th2) [18]. Because T cells from patients with CTCL proliferate in response to anti-CDw60, we asked whether proliferation was associated with release of γ -interferon. T cells from the patients and a healthy control group were incubated with either anti-CDw60 or a mitogenic pair of anti-CD2 antibodies. Anti-CD28 was co-cultured with PMA to obtain optimal stimulation.

Table IV. Immunophenotyping of Peripheral Blood Mononuclear Cells^a

	Patient				Control Subjects
	1	2	3	4	
CD4 ⁺	94	86	85	30	41 $\pm$ 5
CD8 ⁺	2	4	7	19	17 $\pm$ 2
CD2 ⁺	62	12	73	48	69 $\pm$ 4
CD3 ⁺	61	81	72	40	57 $\pm$ 10
TCR ⁺	2	86	71	34	54 $\pm$ 8
CD19 ⁺	3	3	2	1	9 $\pm$ 2
CDw60 ⁺	95	98	85	96	28 $\pm$ 7
CD25 ⁺	0	0	0	3	0 $\pm$ 0
HLA-DR ⁺	4	14	8	37	30 $\pm$ 3
Leu-M3 ⁺	3	3	4	35	15 $\pm$ 2

^a Number of positive cells relative to total cell number.

^b Number of CDw60 positive cells relative to total number of positive T cells determined by CD2 and CD3 staining cells.

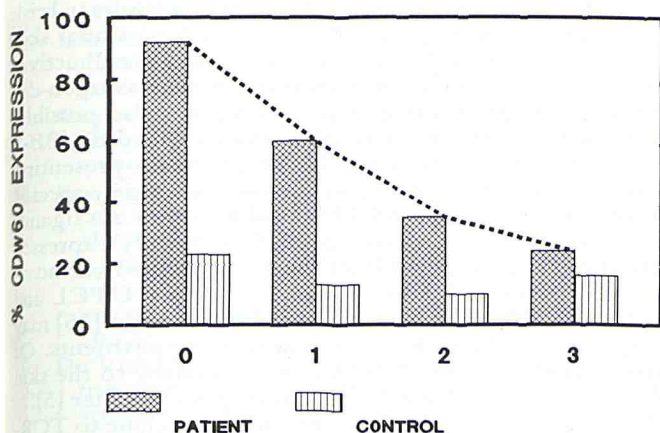
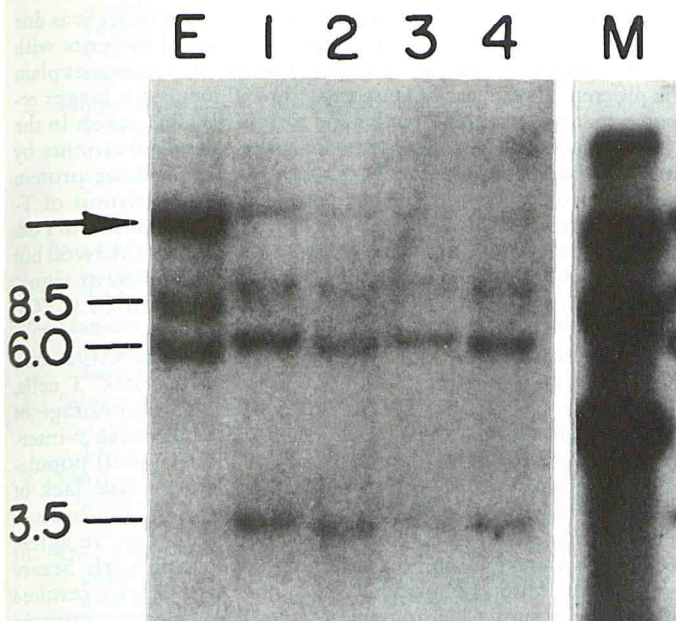


Figure 3. a) Southern blot analysis of a peripheral blood T-cell line obtained from patient 1 (E). The analysis was performed on the cells after 1–4 weeks in culture. DNA samples were digested with *HindIII* and were hybridized to the TCR- β probe to detect TCR β -chain gene rearrangements. Arrow, rearranged band. The malignant clone is gradually replaced by other clones, as indicated by the disappearance of the TCR gene rearrangement. Lane M shows size markers (23, 9.4, 6.6, 4.2, 2.3 kb). b) T-cell lines were initiated from both patient 1 (cross-hatched bars) and a healthy control subject (striped bars). After 1–3 weeks in culture, analysis of CDw60 antigen was performed on a Becton Dickinson fluorescence-activated cell sorter IV flow cytometer. Anti-CDw60 was identified with fluorescein-conjugated goat anti-mouse immunoglobulin. Analysis was performed on 10,000 cells, and the acquired data are expressed as percent of CDw60⁺ cells.

It is surprising that despite the brisk proliferation induced by CDw60, CD2, or CD28, T cells from patients with CTCL did not release detectable amounts of γ -interferon, a secretion pattern that resembles Th2 cells (Fig 6). In contrast, normal blood T cells all demonstrated inducible γ -interferon production (Fig 6).

DISCUSSION

We recently demonstrated that antigen-presenting Langerhans cells in lesional CTCL epidermis are both in an abnormal state of differentiation characterized by an aberrant expression of CD36 and

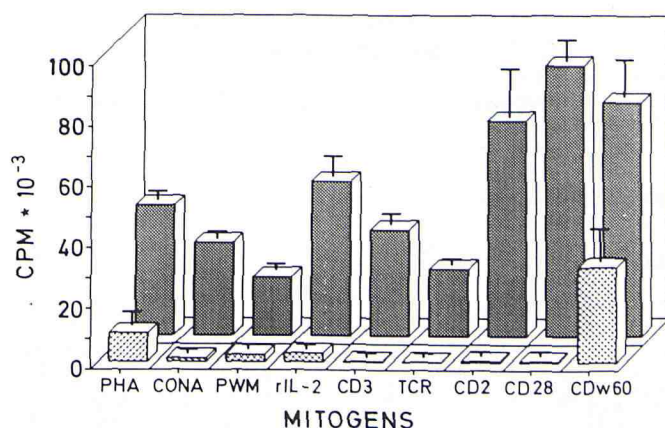


Figure 4. Fifty-thousand MNC from each patient were stimulated with various mitogens and T-cell-activating surface molecules, as single stimuli (cross-hatched bars) or in combination with PMA (stippled bars). The resultant T-cell proliferation was measured as TdR incorporation after 48-h incubation. Data are expressed as mean cpm $\pm$ SEM of experiments with four patients with CTCL.

CD1b [19–21] and in a hyperstimulatory functional state capable of activating autologous CD4⁺ T cells in the absence of added exogenous antigens [4]. Efficient blockade of T-cell proliferation was achieved with a blocking antibody to major histocompatibility complex class II molecules, indicating that the primary signal was transmitted through the TCR [4]. In contrast, attempts to stimulate and propagate malignant T cells *in vitro* from human T-cell lymphotropic virus-1-negative patients with CTCL have been extremely difficult [12–17, 22–25], with a few exceptions [26,27]. We therefore suggest that TIL may be responsive to antigen-receptor signaling provided by hyperstimulatory Langerhans cells present in CTCL epidermis. On the other hand, the malignant clone may be stimulated preferentially through alternative activation pathways, such as CD2, CD28, and CDw60.

Our results in this study demonstrate that the antigen-independent T-cell-activating molecule CDw60 is highly expressed on

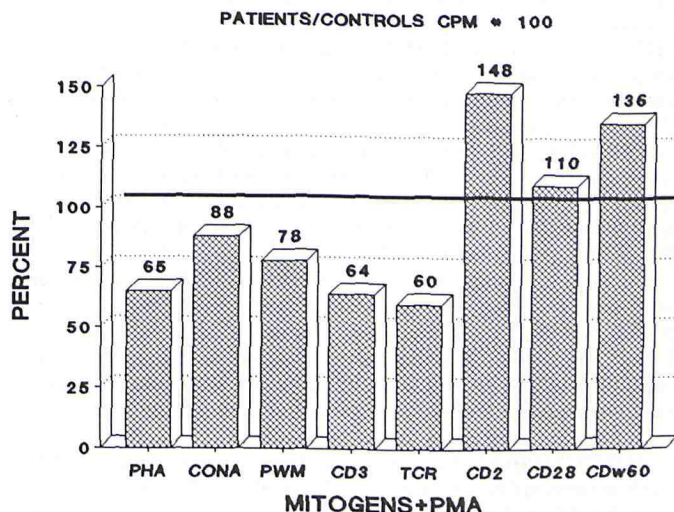


Figure 5. Proliferative response of 50,000 MNC cells from four patients with CTCL compared with that of 50,000 MNC from four healthy subjects. The y-axis demonstrates the percent of TdR incorporation of the CTCL T cells relative to healthy subjects ($n = 4$).

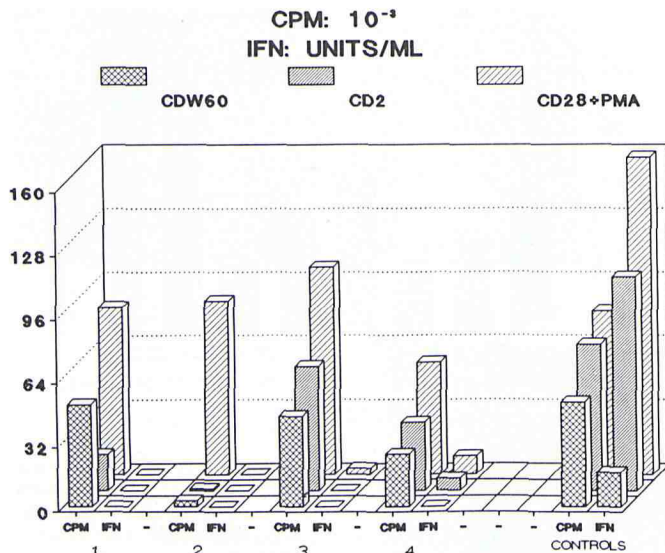


Figure 6. Fifty-thousand MNC from patients with CTCL and a healthy control group were incubated with anti-CDw60, a pair of CD2 antibodies, or anti-CD28. The latter was co-cultured with PMA. The x-axis is split into sections: 1–4, response of each individual patient with CTCL; CONTROLS, group response of normal control subjects. *Left bars* in each section demonstrate the resulting T-cell proliferation measured as TdR incorporation, shown as cpm on the y-axis; *right bars* in each section represent the release of γ -interferon from the T-cell supernatant, measured by an enzyme-linked immunosorbent assay kit and shown on the y-axis as U/ml.

malignant T cells in both the skin and peripheral blood of patients with CTCL (Tables III and IV; Fig 1a,b). In contrast to the high frequency of CDw60 on malignant CTCL T cells, *in vitro*-generated non-malignant TIL in patients with CTCL have a normal level of CDw60 expression (Fig 3b). The diminished expression of CDw60 on TIL was not due to the culture procedure because T-cell clones derived from psoriatic skin maintained a high frequency of CDw60 expression, reflecting high *in vivo* expression [28].

In both normal T cells [5] and synovial fluid T cells [29], CDw60 is expressed on a proportion of all T-cell subsets examined, including CD4⁺, CD8⁺, CD45RO⁺, and CD45RA⁺ T cells. CDw60 appears to be expressed on a somewhat higher percentage of CD45RO⁺ cells than CD45RA⁺ cells among T cells isolated from peripheral blood, but expression on the CD4⁺ and CD8⁺ subsets was comparable. The level of CDw60 expression observed on CTCL cells has not been found thus far on any normal peripheral blood T-cell subsets. Furthermore, malignant T cells that lack CD4 can in some cases express high levels of CDw60, such as variants of the HSB2 leukemic T cell line [5]. In addition, CDw60 is not a marker for T-cell activation because expression of CDw60 is not induced by activation of T cells [5].

The high frequency of CDw60⁺ T cells in CTCL was correlated with a brisk response to stimulation with anti-CDw60 relative to anti-TCR/CD3 and mitogenic lectins. In addition, relative to T cells from healthy individuals, activation of T cells from patients with CTCL by CDw60, CD2, and CD28 resulted in enhanced activation. It could be argued that these findings are due to a preferential activation of CD4⁺CD45RO⁺ T cells relative to CD4⁺CD45RA⁺ by anti-CDw60; however, because anti-CD3 preferentially activates CD4⁺CD45RO⁺ T cells in normal subjects [30], and we found a decreased response to anti-CD3 in patients with CTCL who demonstrate an enhanced number of CD4⁺CD45RO⁺ T cells, a simple numerical explanation cannot account for the observed differences. In normal subjects, CDw60 is mitogenic for peripheral blood T cells only in the presence of accessory cells [5]. It could be argued that the depressed response to

mitogenic lectins and TCR-complex-mediated activation was due to a lack of accessory cells in the peripheral blood of patients with CTCL. However, inadequate accessory cell function cannot explain the differential response because anti-CDw60 induced a larger response than the mitogenic lectins and anti-TCR/CD3, even in the presence of PMA. PMA bypasses accessory cell requirements by directly stimulating calcium and phospholipid-dependent protein kinase-C activity [31] and thereby permits direct assessment of T-cell proliferation capacity. Thus, T cells from patients with CTCL exist in a distinct activation state. Using PMA, not only CDw60 but also other activating molecules, CD2 and CD28, represent signaling mechanisms in the antigen-independent activation of CTCL T cells.

Despite proliferation induced by anti-CDw60, anti-CD2, and anti-CD28, no γ -interferon was produced by the patients' T cells. Because the patients with CTCL expressed a high percentage of "memory" T cells, these results were not expected because γ -interferon is preferentially synthesized by the "memory" T-cell population on stimulation [32]. Our findings may explain the lack of epidermal expression of ICAM-1 in these patients *in vivo* because this molecule is induced by γ -interferon. Our findings are in agreement with a previous report concerning one patient with Sezary syndrome in whom stimulation of leukemic blood T cells resulted in a markedly diminished amount of γ -interferon release compared with healthy subjects [33].

Because stimulation through antigen-independent pathways, such as CDw60, CD2, and CD28, on CTCL T cells results in brisk activation, these pathways may be operative in the lesional skin milieu and may contribute to *in vivo* chronic clonal T-cell activation, even in absence of antigen. Cooperation between antigen-dependent and antigen-independent T-cell activation is also possible. This is highlighted by a recent hypothesis that CD28 and B7/BB-1 are a receptor/ligand pair responsible for an antigen-presenting cell-derived co-stimulatory signal involved in antigen-specific IL-2 production by T cells [34]. LFA-3 and B7, which are ligands for CD2 and CD28, respectively, are clearly abundantly expressed in the skin. The natural ligand for CDw60 is still unknown; however, the presence of CDw60-positive T cells in the CTCL and psoriatic skin, inflammatory synovium, and thymic tissue [35] may be explained by a ligand for CDw60 in these compartments. Of interest, malignant human T-cell lines without affinity to the skin only weakly express CDw60 or lack this antigen altogether [5].

Thus, diminished responsiveness of the malignant clone to TCR-mediated signaling may explain the difficulty many investigators have had in propagating the clone *in vitro* and may also indicate escape from normal regulation. Activation of CTCL T cells via antigen-independent pathways may be a new approach to understand the immunoregulatory events in CTCL.

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