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# Chromosomally Clonal T Cells in the Skin, Blood, or Lymph Nodes of Two Sezary Syndrome Patients Express CD45RA, CD45RO, CDw150, and Interleukin-4, but no Interleukin-2 or Interferon-γ

Leena Karenko, Hanna Nevala, Maria Raatikainen, Kaarle Franssila,* and Annamari Ranki
Departments of Dermatology and *Pathology, Helsinki University Central Hospital, Helsinki, Finland

**Cutaneous T cell lymphomas are considered to represent a clonal malignancy of mature T lymphocytes of the T helper memory subtype. A method enabling the direct identification of clonal malignant cells in tissue and, at the same time, identification of the surface molecules they express has not been available, however. We have developed an application of the FICTION technique (simultaneous fluorescence immunophenotyping and interphase cytogenetics) to be used on fresh blood, skin, and lymph node samples. A prerequisite for this method is the characterization of a moleculocytogenetic clone in order to select the proper probes. With this method, we demonstrate that the true malignant cells express CD3, CD4, and CD45RO in the blood, skin, and lymph nodes of two Sezary syndrome patients. The majority of these cells express also CD45RA (albeit of varying intensity) and CDw150. The cytokine expression pattern of the clonal cells in skin and lymph nodes was interleukin-2 and interferon-γ negative and interleukin-4 positive. Interleukin-10 expression varied. The malignant cells did not express granzyme B, thus indicating that they do not have cytotoxic properties. Clonal cells with the same constant phenotype could be found even in lymph nodes with not yet morphologically identifiable malignant cells. This is the first report of the FICTION method applied directly on skin tissue. With this method we demonstrated that the chromosomally clonal cells in these two cases of Sezary syndrome could be intermediate forms between naïve CD45RA+ and CD45RO+ Th2 cells.** ***Key words: CD45/CDw150/cutaneous T cell lymphoma/extracutaneous. J Invest Dermatol 116:188–193, 2001***

In order to understand the pathomechanism and biology of primary cutaneous T cell lymphomas (CTCLs), the phenotype and function of the malignant cells in various compartments of the body should be known. The malignant lymphocytes in most CTCLs are considered to represent a malignancy of mature T lymphocytes of the T helper memory subtype (Haynes *et al*, 1981; Ralfkiaer, 1991). Previous studies of the phenotype of these cells have been based on morphologic identification of the malignant cells or on flow cytometric identification of clonal T cell receptor (TCR) α, β, or γ locus rearrangement[1] (Haynes *et al*, 1981; Longley *et al*, 1995; Dummer *et al*, 1996; Bakels *et al*, 1997; Bagot *et al*, 1998). Based on functional *in vitro* assays of peripheral blood mononuclear cells, Sezary syndrome (SS), the leukemic subtype of CTCL, has been suggested to present the Th2-type T cell profile whereas mycosis fungoides (MF) would represent a Th1-type profile (Vowels *et al*, 1992; Saed *et al*, 1994; Dummer *et al*, 1996). These methods may not always have identified specifically the true malignant cells, however, as the CTCL lesions also contain clonal T cells reacting against the tumor cells (Bagot *et al*, 1998; Thor Straten *et al*, 1998). Also, our recent studies with fluorescent *in situ* hybridization (FISH) have shown that chromosomal clones can be detected in the blood of CTCL patients even in the absence of morphologically identifiable Sezary cells (Karenko *et al*, 1997).

To reliably identify the membrane markers and cytokines expressed by the true malignant cells, we have identified these cells directly in the tissues with *in situ* hybridization using chromosome centromere-specific probes in combination with simultaneous immunohistochemical staining. A prerequisite for this method is the characterization of the chromosomal clone to be able to select the proper probes. We have modified the FICTION technique (simultaneous fluorescence immunophenotyping and interphase cytogenetics) of Weber-Matthiesen *et al* (1992, 1993) by using new, intense colors visible with a standard (8300) filter set, and, in the study of solid tissue, by using touch preparations instead of cryosections, which allow the observation of intact, whole cells. This is the first report of skin studied with such a method and also the first report where the true malignant cells of CTCL are identified directly in a tissue with immunophenotyping and chromosomal markers.

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Reprint requests to: Dr. Leena Karenko, Skin and Allergy Hospital, P.O. Box 160, FI-00029 HUS, Finland. Email: Leena.Karenko@hus.fi

Abbreviations: FICTION, simultaneous fluorescence immunophenotyping and interphase cytogenetics; FISH, fluorescence *in situ* hybridization; MF, mycosis fungoides; SLAM, signaling lymphocytic activation molecule; SS, Sezary syndrome.

[1]Urban A, Asadullah K, Friedrich M, Volk H-D, Sterry W, Döcke W-D: CD34RA-expressing malignant T-helper cells in Sezary-syndrome (SS). 29th annual meeting of the European Society for Dermatological Research, Montpellier 22–25 September 1999. *J Invest Dermatol* 113:474 1999 (abstr.)

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**Table I. Phenotype of cells with clonal chromosomal aberrations (extra copies of chromosomes 1 and 8[a]) in two patients with Sezary syndrome**

| Tissue | Sample n : o - Patient n : o | Months after CTCL diagnosis | TCR gene rearrangement[i] | Surface marker | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | CD3 | CD4 | CD8 | CD45RO | CD45RA | CDw150 | Granzyme B |
| Blood | 1-1 | 34 | β | +[b] | + | –[c] | 75%[d] | 85%[d] | + | ND[e] |
| | 1-2 | 33 | β | + | + | ND | 85%[d] | dim[f] | ND | ND |
| Skin lesions[g] | 1-1 | 34 | | + | + | – | + | dim[f] | 50%[d] | – |
| | 1-2 | 33 | β. α | + | + | – | + | dim[f] | 50%[d] | < 10%[e] |
| Lymph node, | 1-1 | –6 | | ND | ND | ND | + | + | + | – |
| no lymphoma histology | 1-2 | 12 | | ND | ND | ND | + | + | + | – |
| Lymph node, post mortem | 2-1 | 34 | β | + | + | –[c] | 90%[d] | 90%[d] | + | – |

[a]Karenko *et al*, 1999, and Kähkönen M, personal communication.
[b]All clonal cells positive if not otherwise indicated.
[c]Few weakly positive cells.
[d]Percentage of clonal cells positive for the marker.
[e]ND, not done.
[f]Weakly positive cells.
[g]Both samples negative for CD30 antigen.

## MATERIALS AND METHODS

**Patients and tissue samples** The material consisted of tissue samples obtained from two patients who developed SS and showed a dominant clone of malignant cells with supernumerary copies of chromosome 8, in near-diploid or in near-tetraploid cells in blood (Karenko *et al*, 1999; see also below). The clones contained also other complex chromosomal abnormalities, which were partly different in the two patients but remained remarkably stable during the course of the disease.

Case 1 was a 64-y-old male patient, when he became erythrodermic after mild psoriasis, localized to elbows, knees, and the scalp. In a skin biopsy atypical CD4-positive T lymphocytes were seen in the upper dermis but the biopsy was regarded as nondiagnostic of CTCL because no epidermotropism was seen. There were 10% of Sezary cells in the blood. Six months later, enlarged lymph nodes were detected in his left armpit and, in a biopsy of such a lymph node, nonspecific hyperplasia was seen. In some areas the paracortical area (T zone) was expanded but no definite lymphoma was seen (specimen L 1-1 in the FICTION study). The patient was then treated with psoralen and ultraviolet A. After an additional 6 mo, Pautrier's microabscesses were seen in a skin biopsy, and the histologic diagnosis of MF was made. One month later, 25% of Sezary cells were found in his blood, and the diagnosis of SS was made (T4N0B1). His cultivated blood lymphocytes showed 45% of clonally very aberrant mitoses in G-banding. Further studies showed a gain of chromosome 8 in comparative genomic hybridization, and supernumerary copies of chromosome 8 in near-diploid or near-tetraploid cells in FISH (Karenko *et al*, 1999). In computerized tomography, enlarged lymph nodes were detected in armpits and groin. He was then sequentially treated with etretinate, total skin electron beam irradiation, and cladribine. The disease progressed, and the treatment was discontinued after 6 mo (18 mo after the first biopsy) because of thrombocytopenia. He died 2 y and 10 mo after the diagnosis of CTCL. A blood specimen (B 1-1) taken 1 wk before death showed clonal cells similar to those detected 1 y 10 mo earlier (25 aberrant out of 25 mitoses after phytohemagglutinin stimulation). This blood sample and postmortem skin (S 1-1) and lymph node specimens (L 2-1) were used for the modified FICTION.

Case 2 was a 52-y-old male patient who presented with an eczematous dermatitis on his soles, ankles, breast, and back. Four months later, a skin biopsy showed typical histology of MF, and he had 30% of Sezary cells in peripheral blood (T2N0B1). Clinically, he had patch stage skin lesions, and he was treated with psoralen and ultraviolet A. Ten months after the diagnostic skin biopsy, he developed erythrodermia, and the diagnosis of secondary SS was made. A blood sample showed a near-tetraploid aberrated mitosis (1 of 100 mitoses), that later was observed to be clonal. Two months later, his groin lymph nodes were enlarged and showed histologically dermatopathic lymphonoditis with a slight suspicion of malignant (T4N1B1) affision. This biopsy (L 1-2) was studied with the modified FICTION. He was then treated with whole body and local electron beam irradiation (2000 Gy and up to 20 Gy, respectively), cladribine, and, finally, chlorambucil. In blood samples taken 2 y 7 mo after the diagnosis of MF, and 2 mo thereafter (B 1-2), and in a skin tumor biopsy taken at the latter time point (S 1-2), very similar cytogenetic or moleculocytogenetic findings were detected (Karenko *et al*, 1999). They included gain of chromosome 8 in comparative genomic hybridization (blood and skin tumor) and supernumerary copies of its centromere in FISH both in near-diploid cells and in the last sample also in near-tetraploid cells (blood). The latter blood (B 1-2) and skin samples (S 1-2) were examined with the modified FICTION method. The number of Sezary cells in the latter blood specimen (B 1-2) was 95%, and 95% of phytohemagglutinin-induced mitoses (19 of 20 mitoses) showed the clonal aberrations. He died 2 y 10 mo after the diagnosis of MF.

Blood lymphocyte preparations of three healthy, nonsmoking persons were used as controls. Sections of frozen and formalin-fixed paraffin-embedded skin lesion biopsies of one patient with histopathologically confirmed lymphomatoid papulosis, and with a favorable course, were examined for granzyme B positivity (see below).

**Preparations and immunohistologic staining** Cytospin preparations of Ficoll-enriched blood mononuclear cells or touch preparates of frozen skin or lymph node biopsies were fixed in ice-cold acetone, and an immunohistologic staining was performed using antibodies to CD3, CD4 (Dako, Glostrup, Denmark), CD45RA (Caltag, Burlingame, CA), CD45RO, CD8 (Dako), granzyme B, interleukin-2 (IL-2), IL-4, IL-10 (Santa Cruz Biotechnology, Santa Cruz, CA), interferon-γ (IFN-γ) (Neomarkers, Fremont, CA), and signaling lymphocytic activation molecule (SLAM, CDw150; A12 antibody kindly provided by Dr. G. Aversa, DNAX Research Institute of Molecular and Cellular Biology, Palo Alto, CA). The primary antibodies were monoclonal mouse antibodies except for the polyclonal rabbit antibodies against CD3 and IL-2, and goat antibodies against granzyme B, IL-4, and IL-10. The secondary antibodies were antirabbit or antimouse goat conjugates of Texas Red X or Alexa 594 R (Molecular Probes, Leiden, The Netherlands), or antirabbit, antigoat, or antimouse donkey conjugates of Rhodamine Red X (Jackson Immuno Research Laboratories, West Grove, PA). When necessary, for detection of weak mouse primary antibodies, Rhodamine Red X conjugate was followed with antirhodamine rabbit (Molecular Probes) and an antirabbit donkey conjugate of Rhodamine Red X (Jackson Immuno Research Laboratories). All antibody layers were preceded by a layer of normal serum from the animal species, in which the secondary antibody was raised. Through every step of the process, each slide was accompanied by a similar control slide with no primary antibody.

***In situ* hybridization** After immunohistologic staining, the samples were hybridized with a chromosome 8 centromere-specific probe (D8Z2[pJM128]) labeled (Karenko *et al*, 1997) with a green fluorescent color (Alexa 488, Molecular Probes) or digoxigenin, or a chromosome 1 centromere-specific probe (1q12[pUC177]) labeled with biotin (both probes were kindly provided by Dr. T. Visakorpi, University of Tampere, Finland). The hybridization was performed as previously described (Karenko *et al*, 1997). After posthybridization washes, the biotin-labeled probes were detected with deglycosylated avidin conjugated with a blue color (NeutrAvidin conjugated with Alexa 350 or Cascade Blue; Molecular Probes), followed by biotinylated antiavidin (goat, Vector Laboratories, Burlingame, CA, or mouse, Sigma, St. Louis, MO), and a new layer of the same deglycosylated avidin conjugate. The digoxigenin-labeled probes

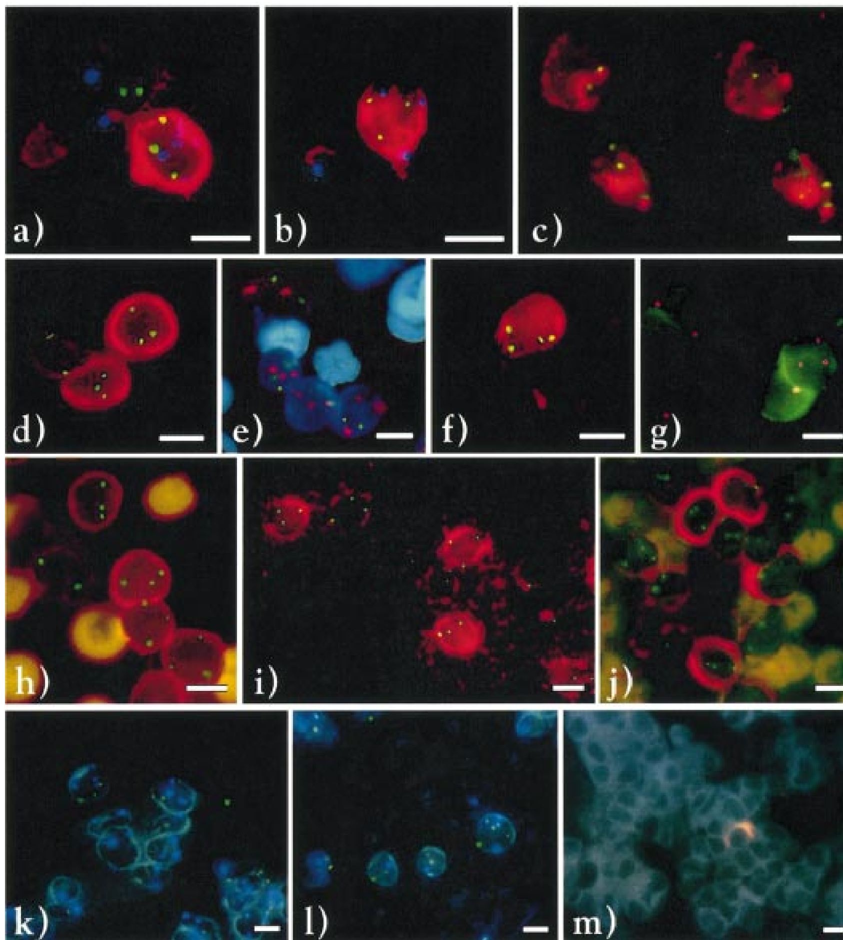


**Figure 1. The phenotype of clonal malignant cells in CTCL is detectable with a modified FICTION method in blood, skin, and lymph node samples.** Chromosome 8 centromere is visualized with green fluorescence (Alexa 488) in all panels except for (*g*), where it is red (Alexa 495). Chromosome 1 centromere is visualized blue in (*a*) and (*b*), but red in (*e*). Panels (*a*)-(*e*), (*g*)-(*i*), and (*l*) show tissues of case 1, and (*f*), (*k*), and (*m*) of case 2. (*a*) CD3-positive (Texas Red X) clonal blood cell together with a normal CD3-negative cell; (*b*) similar CD-3-positive phenotype of a clonal cell in the lymph node (L 2-1); (*c*) CD4-positive (Rhodamine Red X) clonal cells in the skin shown in two focuses. CD45RO-positive (Texas Red X) clonal cells along with a normal CD45RO-negative cell in (*d*) a lymph node (L 2-1), (*e*) the blood (CD45RO dark blue, Alexa 350; red blood cells stain light blue), and (*f*) a skin lesion (Rhodamine Red X) and (*g*) a dermatopathic lymph node (L 1-1, green FITC). CD45RA-positive (Texas Red X) clonal malignant cells (*h*) in the blood along with a normal, CD45RA-negative cell and (*i*) in a lymph node (L 2-1) along with one CD45RA-negative malignant cell and several normal CD45RA-negative cells. (*j*) CDw150/SLAM-positive (Texas Red X) and negative clonal cells in the blood along with normal cells. (*k*) Granzyme-B-negative clonal and normal cells in a lymph node (L 1-2) with no morphologic lymphoma involvement; (*l*) control staining without antigranzyme antibody (L 1-1), and (*m*) a rare granzyme-B-positive cell (Rhodamine Red X) in a similar lymph node of case 2 (L 1-2). *Scale bars*: 10 μm.

were detected with sheep antidigoxigenin antibody (Roche Molecular Biochemicals, Mannheim, Germany), followed by donkey antibody conjugated with fluorescein isothiocyanate (FITC) (Jackson Immuno Research Laboratories).

Alternative stainings were used to confirm the results. The immunohistology was performed by detecting the primary mouse antigen with biotinylated antimouse raised in horse, followed by avidin conjugated with FITC (Vector Laboratories), and hybridization with a chromosome 8 centromere-specific probe labeled with a red color (Alexa 594, Molecular Probes). Alternatively, the primary mouse antibody was detected with rabbit antimouse antibody (Sigma), followed by antirabbit raised in swine (Dako), antiswine raised in rabbit (Rockland, Gilbertsville, PA), and finally goat antirabbit conjugated with a blue color (Alexa 350, Molecular Probes). The probes used were chromosome 8 centromere-specific probe labeled with Alexa 488 and chromosome-1-specific probe labeled with Texas Red X (colors, Molecular Probes).

The formalin-fixed, paraffin-embedded skin biopsies of cases 1 and 2 were examined for CD30 and a skin biopsy of the third patient with lymphomatoid papulosis for granzyme B with the standard immunoperoxidase technique (for CD30, StreptABC Complex/HRP kit, Dako, followed by 3-amino-ethylcarbazole, for granzyme B).[2]

**Analysis and imaging** Preparations were analyzed with an ultraviolet microscope (Olympus BX 50, Tokyo, Japan; equipped with filter set 8300 and tripleband exciter 83103x, Chroma Technology, Brattleboro, VT). Pictures were taken with a cooled CCD camera (Sensi Cam, PCO, Computer Optics, Kelheim, Germany) connected to a computer (Dell, Limerick, Ireland; with software Image pro Plus, Media Cybernetics, Silver Spring, MD).

## RESULTS

**Immunophenotype of chromosomally clonal cells** A similar and constant phenotype over time was found in malignant cells with clonal chromosomal aberrations (i.e., supernumerary copies of chromosome 8 in near-diploid or near-tetraploid cells) in the blood, skin, and lymph nodes of two SS patients (**Table I**). The malignant cells were CD3 and CD4 positive but CD8 negative (**Table I** and **Fig 1*a–c***). Both CD45RA and CD45RO positivity was observed in the majority of clonally malignant cells in both patients, although in the blood of both patients and in the skin of case 2 the staining with CD45RA was of weaker intensity (**Table I**, **Fig 1*d–i***). In the postmortem lymph node of case 1 (L 2-1), both antigens showed intense staining in the clonal cells (**Fig 1*d*, *i***).

The lymph node biopsies of both patients obtained before histologically verified lymphoma involvement showed chromosomally clonal cells in hybridization (25%–35% of the cells in the touch preparate in L 1-1, and 24%–42%, respectively, in L 1-2). In both cases, the clonal cells expressed both CD45RA and CD45RO markers (**Table I, Fig 1*g***).

The CDw150 (SLAM) antigen, characterizing activated memory T cells (CD45ROhigh) was detected in all tissues of case 1 (**Table I**). In the skin of both patients, the expression was observed in about half of the clonal cells (**Fig 1*j***). In contrast to positive control samples (blood lymphocytes of a healthy person, and the patient with lymphomatoid papulosis), granzyme-B-positive cells were observed only occasionally in the skin or lymph node samples of case 1, and none of these cells represented the malignant clone (**Fig 1*k–m***). A few (less than 10%) clonal skin cells of case 2 expressed granzyme B very weakly (**Table I**).

[2]Nevala H, Karenko L, Ranki A: Pro- and antiapoptotic markers in cutaneous T-cell lymphoma (CTCL) skin infiltrates and in comparison to lymphomatoid papulosis. Submitted.

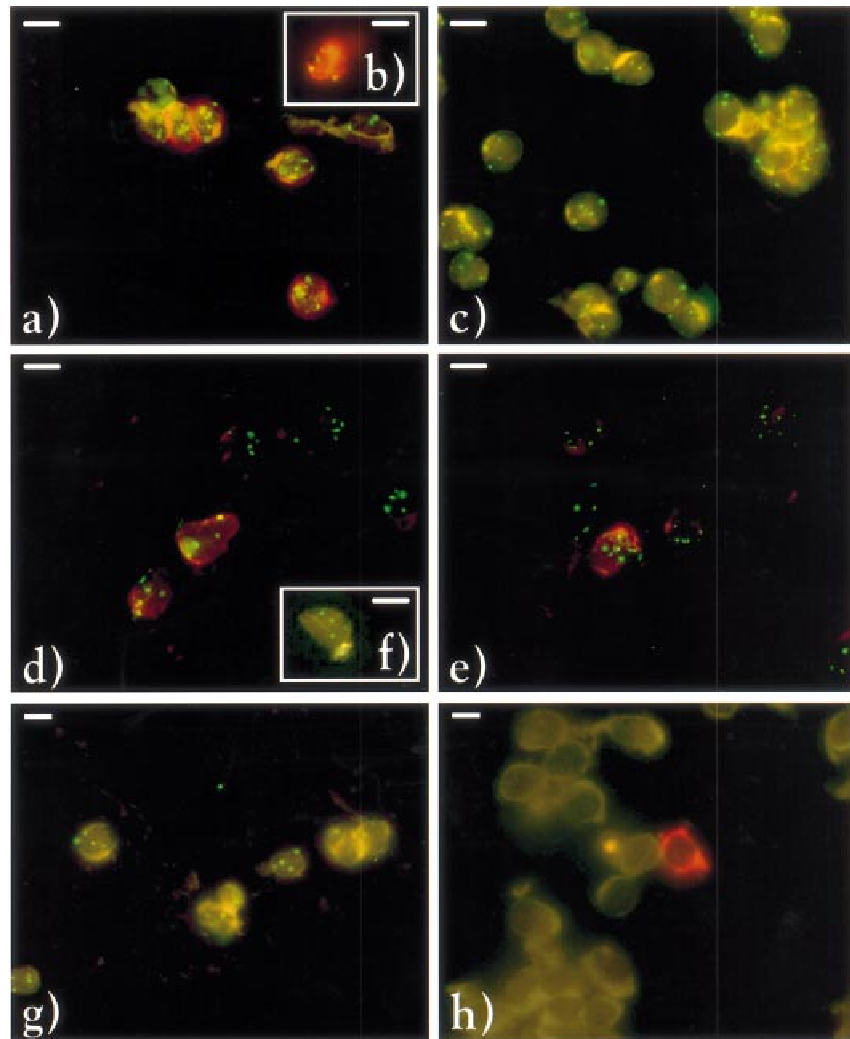


**Figure 2. The majority of chromosomally clonal cells expressed IL-4, and the cells of case 2 expressed IL-10, but the expression of IL-2 was rare and no IFN-γ was expressed by the malignant cells.** Chromosome 8 centromere is visualized with green fluorescence (FITC) in all panels except for (*h*). Panels (*a*)–(*c*), (*g*), and (*h*) show lymph node tissue of case 1 (L 2-1), and (*d*)–(*f*), skin tissue of case 2 (S 1-2, all photographed with color camera). (*a*) IL-4-positive (red, Alexa 594) clonal cells together with normal IL-4-negative cells; (*b*) a brightly IL-4-positive clonal cell (red, Alexa 594); (*c*) control staining without anti-IL-4 antibody (no red signal); (*d*) IL-10-positive (red, Alexa 594) and negative clonal cells; (*e*) an IL-2 positive clonal cell (red, Rhodamine Red X) and IL-2-negative clonal cells; (*f*) a control staining without primary antibody; (*g*) IFN-γ-negative clonal cells; (*h*) a rare IFN-γ-positive cell in a plain immunostaining of the same tissue as in (*g*). *Scale bars*: 10 μm.

**Table II. Chromosomally clonal cells in two patients with Sezary Syndrome expressed IL-4, but the expression of IL-10 was variable between the patients**

| Tissue | Sample n : o | IL-2 | IL = 4 | IL-10 | IFN-gamma |
|---|---|---|---|---|---|
| Skin lesion | 1-1 | +3%[a] | ++50%, +50% | +5% | ND[b] |
| | 1-2 | +2% | +80% | +80% | 0% |
| Lymph node, post mortem | 2-1 | +2% | ++25%, +75% | +8% | 0% |

[a]+, positive immunostaining; ++, brightly positive immunostaining.
[b]ND, not done.

**The cytokine expression** The majority of clonal cells expressed IL-4 (**Fig 2**). The staining intensity was variable with all colors used (Rhodamine Red X, Alexa 594 R), showing very bright color intensities in up to 50% of the clonal cells. At most 20% of the clonal cells were IL-4 negative (**Table II**). The majority of the clonal cells of case 1 were IL-10 negative (**Table II**), but the majority of the cells of case 2 were IL-10 positive (**Table II**, **Fig 2**). The majority of the clonal cells of both patients were IL-2 negative and none expressed IFN-γ (**Table II**, **Fig 2**). Thus, the cytokine expression pattern of the clonal cells in skin and lymph node was IL-2 negative, IL-4 positive, variable for IL-10, and IFN-γ negative.

## DISCUSSION

Phenotypically, Sezary cells have been considered to be CD4+, CD45RA-, CD45RO+, and functionally of Th2 type (Vowels *et al*, 1992; Saed *et al*, 1994; Dummer *et al*, 1996). The cells of our patients commonly expressed also CD45RA, however, normally observed in naive T cells (Clement *et al*, 1988). They also expressed SLAM (CDw150), which is normally expressed on CD45RO+ peripheral blood memory cells and rapidly upregulated on activated T cells (Cocks *et al*, 1995). SLAM directs the immune response towards the Th0-Th1 pathway (Aversa *et al*, 1997).

Normally, naive CD45RA+ cells express IL-2, and mature CD45RO+ cells express IL-4, IL-5, IL-10 (Th2), or IFN-γ (Th1). A strong coexpression of both CD45 isoforms occurs during transition from CD45RA+ to CD45RO+ (Dbright in flow cytometry), with expression of IL-2 and IFN-γ (LaSalle and Hafler, 1991; Picker *et al*, 1993; Hamann *et al*, 1996). Cells with weaker coexpression of CD45RA/RO (Ddull) express mainly RO+ type cytokines, IL-4, IL-5, IL-10, or IFN-γ, and have been suggested to represent some stage in T cell differentiation or resting primed T cells (Hamann *et al*, 1996). The differentiation of naive to Th1 or Th2 cells seems to go through a phase where naive cells express small amounts of IL-4 with IL-2 (Kamogawa *et al*, 1993; Bullens *et al*, 1999). The chromosomally clonal cells of our patients characteristically expressed IL-4, typical of RO+ type 2 Th cells, but only in one case (case 2) was IL-10 expression observed. Despite the variable expression of CDw150, the clonal cells of our patients did not express IFN-γ, usually upregulated by CDw150 (Cocks *et al*, 1995). Taken together, the phenotype CD45RA+, CD45RO+, CDw150±, IL-4+, IL-2, IFN-γ, and with the variation in IL-10 expression between the patients, the clonal cells seem to be intermediate forms between naive CD45RA+ and CD45RO+ Th2 cells. Possibly, they might represent cells that have not attained complete maturity, or they could be mature Th2 cells partly reverted towards a more naive or resting T cell type (Hamann *et al*, 1996). The differences in the immunohistologic staining intensity of IL-4-positive cells must be interpreted with caution, and the interactions with other cells of the microenvironment were not studied. Our finding of malignant cells with the phenotype CD45RA+, CD45RO+ is in concordance with the heterogeneity of RA+, RA+/RO+, and RO+ expression between four SS patients studied with flow cytometry by Urban *et al*.[1]

Interestingly, the IL-4 positivity may contribute to the low frequency of apoptotic cells found in skin lesions of CTCL patients,[2] as IL-4 blocks the action of caspase 3 (Manna and Aggarwal, 1998), and IL-4-producing T cells have been shown to be resistant to activation-induced apoptosis (Carbonari *et al*, 2000). In contrast to Vermeer *et al* (1999), who showed cytotoxic T-lymphocyte-associated granzyme B and cytotoxic-granule-associated TIA protein expression in morphologically identified malignant cells in MF patients, we did not find notable granzyme B expression in our SS patients in this or our previous study,[2] although the sample of lymphomatoid papulosis showed clear positive staining.

Our finding of abundant amounts of malignant chromosomal clone cells in lymph nodes with dermatopathic lymphonoditis histology, and obtained even several months prior to malignant infiltrate in the skin, suggests that malignant clones may rise extracutaneously and they may invade lymph nodes early in the course of the disease. It is noteworthy that the SS of one patient evolved from MF. The concept of an early systemic nature of CTCL has gained evidence also from some previous studies (Whang-Peng *et al*, 1982; Veelken *et al*, 1995; Dommann *et al*, 1996; Trotter *et al*, 1997; Tok *et al*, 1998), and early extracutaneous T cell chromosomal aberrations or T cell clonality have been verified even in large or small plaque parapsoriasis more recently (Karenko *et al*, 1997; Muche *et al*, 1999). Thus, lymph node biopsies should be obtained during the early phases of CTCL, possibly even prior to clinical enlargement, to demonstrate the presence of malignant cells. A prerequisite for the use of FICTION in the routine study of lymph nodes or other tissues of CTCL patients, however, is the definition of clonal chromosomal or gene level aberrations suitable for *in situ* hybridization for identification of the malignant clone.

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