INVESTIGATIVE REPORT

Skin-homing CD8+ T lymphocytes Show Preferential Growth *in vitro* and Suppress CD4+ T-cell Proliferation in Patients with Early Stages of Cutaneous T-cell Lymphoma

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A total of 27 T-lymphocyte cell strains were established from skin biopsies of 24 patients with various stages of cutaneous T-cell lymphoma (CTCL) by addition of the T-cell growth factors interleukin (IL)-2 and IL-4. Cellular proliferation and phenotypic changes were measured over 3 months in culture, and T-cell clones were studied using T-cell receptor-γ re-arrangement techniques. An average outgrowth of 134 million T lymphocytes from a 4-mm skin biopsy was observed over 2 months. Initially, most T cells expressed the CD4+ phenotype. In 17 cell strains from patients with early CTCL a statistically significant predominance of CD8+ T-lymphocytes developed over 8-weeks' culture, indicating that CD8+ T cells controlled the growth of CD4+ T cells, whereas CD4+ T cells were predominant in cell strains from advanced CTCL (*p* <0.05). TCR-γ re-arrangement studies revealed, on average, 12 T-cell clones per cell strain, which was reduced over time to 6 T-cell clones per cell strain. Lymphocytes from peripheral blood could kill lymphocytes from an autologous cell strain, suggesting the presence of autoreactive cytotoxic T cells. Our study suggests how skin-homing CD8+ T lymphocytes from patients with early stage CTCL can suppress the *in vitro* growth of skin-homing CD4+ T-lymphocytes, indicating immune surveillance. *Key words: clones; cytotoxicity; mycosis fungoides; parapsoriasis; phenotype; T-cell receptor gamma rearrangement.*

Cutaneous T-cell lymphoma (CTCL) is a group of primary cutaneous lymphomas for which approximately 20–25% of patients have fatal outcome (1–4). An aggressive course of the disease is associated with skin tumours, involvement of lymph nodes and the presence of T-cell clones in the blood. Patients with milder disease survive longer (1, 3–6). This suggests that the immune system in the majority of patients can control the growth of malignant T cells (7–9). Up to 75% of patients, even in the early stages of the disease, have T-cell clones, as measured by T-cell receptor gamma (TCR-γ) re-arrangement analysis (10–12). Telomerase activity is increased and telomere length is reduced in peripheral blood mononuclear cells (PBMC) of patients with the early stages of CTCL, indicating an increased lymphocyte turn-over in the peripheral immune system (13).

This study examined skin-homing T lymphocytes from patients with various stages of CTCL by culturing skin T cells for up to 3 months. The proliferation capacity, immunophenotype and CD4+:CD8+ cell ratio were studied during culture. T-cell clones were detected by studying TCR-γ re-arrangements and genotyping of TCR-γ genes. Results show that T-cell strains can be established from skin biopsies of CTCL patients, and that T-cell strains from patients with early and quiescent disease develop a predominance of CD8+ T cells during *in vitro* growth. Preliminary experiments revealed how autologous blood lymphocytes show cytotoxicity towards skin-homing T cells. Thus, patients with clinically quiescent early-stage CTCL exhibit signs of immune surveillance, whereby the immune system controls growth of the malignant transformed cells.

PATIENTS AND METHODS

Patients

Twenty-four native Saudi patients with an established clinical and histological diagnosis of CTCL were studied, 18 men and 6 women (ratio 3:1), age range 17–65 years, median age 40 years. Fifteen patients showed less than 30% skin involvement including 11 with ≤10% and no lymph node or bone marrow involvement. These patients had mycosis fungoides (MF) patch stage (1). Four patients had MF stage II to IVb with 15–100% skin involvement, 2 of whom had skin tumours and one lymph node involvement. Four were erythrodermic and one patient had fatal T-cell lymphoma with involvement of skin, kidneys and bone marrow. Disease duration was 1–10 years, median 5 years. Some patients were in treatment with PUVA or nitrogen mustard, while others were untreated. This study was approved by the Research Advisory Council, King Faisal Specialist Hospital and Research Centre (project # 990 012).

Histology

A 4-mm skin punch biopsy was taken from an area of affected skin using routine methods. A histological scoring system noted degree of infiltrate in: (*i*) epidermis, (*ii*) papillary and (*iii*) reticulate dermis, and (*iv*) degree of pleomorphism. Each parameter was given a score of 0, 1, 2 or 3, giving a maximum score of 12. One pathologist (BH) evaluated all biopsies without knowledge of the results from cell cultures (Table I).

In vitro culture of cell strains

Skin biopsies from clinically affected skin were placed in sterile physiological saline and transferred to Nunclon® (NUNC, Roskilde, Denmark) 25 cm³ tissue culture flasks with 10 ml pre-warmed RPMI 1640 with 10% human heat-inactivated AB serum, antibiotics and interleukin 2, 1.000 units/ml (IL-2; Proleukin; Chiron) and interleukin 4, 250 units/ml (recombinant human IL-4, Schering-Plough, NJ, USA) and incubated at 37°C at 83% humidity with 6.1% CO_2. No feeder cells or mitogens were added.

A cell strain (or cell line) was regarded as established if cell growth occurred within a 3-week period. The skin tissue was discarded after 2 weeks. Cell density was kept at around

Table I. *Sex, age, clinical diagnosis, histological score and CD4+: CD8+ ratio in cell strains from patients with cutaneous T-cell lymphoma (or mycosis fungoides). The CD4+:CD8+ ratio was determined during the first month of growth (2–4 weeks), and the final value taken from 8–12 weeks of growth. Several cell strains were analysed 3–5 times during culture*

MR #	Sex	Age	Diagnosis	Histology score	CD4+:CD8+ ratio First value (2–4 weeks)	Final value (8–12 weeks)
1	M	42	MF I < 10%	1	1.37	1.14
6	M	25	MF I < 10%	na	6.0	1.11
17	M	44	MF I 10%	na	4.1	normal
41	F	40	MF I < 10%	na	4.4	0.72
163	F	56	MF I < 10%	4	0.23	0.01
443	M	24	MF I < 10%	1	0.18	0.25
446	M	34	MF I 20%	7	na	0.38
449	F	17	MF I < 10%	3	1.83	0.07
131	M	39	MF II 10%	4	0.18	0.01
340	F	50	MF II 15%	na	5.53	0.18
405	M	35	MF II < 10%	7	0.04	0.03
403	M	20	MF II 15%	6	0.18	0.07
428	M	50	MF II 10%	5	0.09	0.54
369	M	42	Erythroderma	5	0.28	0.18
365	M	65	Erythroderma	1	0.46	0.08
449	M	65	Erythroderma	4	18.6	0.13
393	M	29	Erythroderma	3	0.1	na
Mean				3.76*	2.72	0.33*
433	F	25	MF I 30%	5	6.76	2.74
274	F	40	MF I < 10%	4	7.15	5.37
164	M	49	MF IVa 10%	7	3.16	3.20
295	M	29	MF Ivb 80%	7	18.5	24.0
405	M	50	MF III 100%	11	0.25	32.6
425	M	38	MF II 80%	4	0.66	98.0
438†	M	57	T lymphoma	7	2.11	2.29
				7.85*	5.51	24.0

MR #, medical record number. Patients were subdivided into 2 groups according to whether their CD4+:CD8+ ratio in the last studied culture was less or above an index of 2. Following this it is evident that patients with a low CD4+:CD8+ ratio in their cell strains had a significantly average histology score in parallel with significantly more CD8+ T-cell outgrowth in their cell strains. *$p < 0.05$; Wilcoxon rank sum test. na, not available as 4 biopsies came from patients initially seen outside of our hospital, where the diagnostic biopsy was not available for evaluation. †This patient died.

$1–3 \times 10^6$ per ml. The viability of the cells was estimated using the trypan blue dye exclusion test. Over time the total number of living cells in the culture was recorded using a cell counter (Coulter Counter ZM). Cells were removed from the cultures for proliferation assays, phenotypic analysis, T-cell receptor studies, etc. All analyses were done during the time period when the cell strain showed increasing number of cells in the culture. Outgrowth of fibroblasts was never encountered.

Phenotype analysis

A panel of commercial monoclonal antibodies against various surface markers was used for phenotype analysis according to the World Health Organization (WHO) classification. The following antibodies were from Becton-Dickinson (San Jose, CA, USA): CD3 FITC, CD3 PE, CD3/CD4, CD3/CD8, CD3/16+56, CD3/HLA-DR, CD3/CD19, CD8 FITC, CD8 PE, CD16+56, CD38 PE, CD45 FITC, CD45RA FITC, CD45RO PE, TCR-α/β FITC, TCR-γ/δ PE and isotype controls. The following antibodies were from BD PharMingen, (San Diego, CA, USA): CD40 PE, CD154 PE, CLA FITC, HLA-A,B,C PE (class I) and Beckman Coulter Inc., (Fullerton, CA, USA) delivered the CD38 FITC antibody. Approximately 1×10^6 cells were washed once in phosphate-buffered saline (PBS) 2% foetal calf serum (FCS) prior to staining. The cells were incubated with saturating concentrations of the appropriate monoclonal antibody in PBS 2% FCS for 30 min at 4°C in the dark. After 2 washes the cells were resuspended in PBS 2% FCS and analysed. The samples were analysed on a FACScan flow cytometer (Becton-Dickinson). The CELLQuest software (Becton-Dickinson) was utilized for data acquisition and analyses.

DNA measurements were performed in which cells were treated with a DNA staining kit (Beckman Coulter Inc.) without prior fixation steps, according to the manufacturer's guidelines. Cell cycle analysis was carried out on a FACScan flow cytometer (Becton-Dickinson) equipped with a pulse processing facility to enable discrimination of cell doublets. To estimate the distribution of DNA content in G0/G1, S-phase and G2/M, the post-acquisition software, ModFit LT (Verity Software House, Inc.) was used.

T-cell receptor re-arrangement studies

Genomic DNA was extracted using TRIzol Reagent (Gibco BRL, Life Technologies) according to the manufacturer's instructions.

Polymerase chain reaction (PCR) primer. TCR primers included V2, 3, 4, 5, 8, 9, 10 and 12 for the variable region and JGT3, 4, 12 for the J segment. All V region primers were labelled with Fam, Tet or Hex, respectively. The primer sequences and dyes are shown in Table II. These primers were used as 3 mixes: mix I contained TCR V2, 3, 4; mix II contained V5, 10; mix III contained TCR V8, 9, 12; and the J mix contained JGT3, 4, 12.

Fluorescent PCR. PCR was performed according to a previously published method (14) with a few modifications. Briefly, 1 unit of Taq polymerase (Amplitaq Gold, Perkin Elmer-Cetus, Norwalk, CT, USA) was added to a 25 μl reaction containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl₂, 0.01% (w/v) gelatin, 0.1 mM of each of deoxyadenosine triphosphate, deoxycytidine triphosphate, desoxythymidine triphosphate and deoxyguanosine triphosphate, 20 ng of each primer and 0.5 μg of template DNA.

For amplification of the TCR gene, primers were mixed as indicated above and PCR performed with primers from each V region mix to J mix. Forty-five cycles were carried out at 94°C for 1 min, 60°C for 1 min and 72°C for 1 min. Each PCR was preceded by denaturation at 94°C for 10 min and followed by a final extension of 20 min at 72°C. The PCR products were

Table II. *Primer sequences*

Region	Primer sequences 5' to 3'	Dye
V2	CTTCCTGCAGATGACTCCTACAACAACTCC AAGGTTG	Fam
V3	CTTCCTGCAGATGACGTCTCCACCGCAAGG GATG	Hex
V4	CTTCCTGCAGATGACTCCTACACCTCCAGC GTTG	Tet
V5	TTCCTGCAGATGACGTCTCCAACTCAAAG GATG	Fam
V8	CTTCCTGCAGATGACTCCTACAACTCCAGG GTTG	Hex
V9	GGNACTGCAGGAAAGGAATCTGGCATTCCG	Tet
V10	CTCTGCAGAATCCGCAGCTCGACGCAGCA	Tet
V11	CACTGCAGGCTCAAGATTGCTCAGGTGGG	Hex
V12	ACTCTGCAGCCTCTTGGGCACTGCTCTAAA	Fam
JGT3	AGTTACTATGAGCYTAGTCCC	
JGT4	TGTAATGATAAGCTTTGTTCC	
JGT12	AAGTGTTGTTCCACTGCCAAA	

Primers used for sequencing were non-labelled.

screened by evaluation on ethidium bromide 2% agarose gels. In all experiments negative and normal controls were analysed along with test samples.

Genotyping of TCR-gamma genes

Samples were diluted 1:10 or 1:20 according to the estimated concentration of the PCR product. 3 μl of diluted fluorescently-labelled PCR product was placed in a 1.5 ml Eppendorf tube, vacuum-dried for 10 min and resuspended in 3 μl of loading dye containing tetramethylrhodamine 500 size standard (Applied Biosystems). After denaturation at 95°C for 3 min the samples were chilled quickly on ice and loaded on a 6% acrylamide gel containing 8.3 mol/l urea and electrophoresed for 8 h in a 373A automated sequencer (Applied Biosystem) at a constant power of 30 Watts. The ABI GeneScan Software (Applied Biosystem) was used for data collection and analysis.

T-cell receptor sequencing of skin tumours and cell strains

DNA was extracted from 3 independent tumours from the same patient. Data from genotyping showed that the resulting PCRs exhibited a multi-clonal profile at low background, but with distinct peaks for V2 and V4 forward primers as these were differentially labelled with Fam, Hex and Tet (V2, V3 and V4, respectively). Based on this information it was decided to attempt to amplify tumour DNA from the 3 different sites using unlabelled V2 with JGT 3, 4 and 12, and V4 with JGT 3, 4 and 12 as 6 different PCRs in an attempt to isolate and characterize the predominant clones. Out of the 6 possible products, only V2-JGT 4 did not give any product. The remaining 5 PCRs all gave single bands which were purified using the MicroSpin S-300 HR columns (Amersham Pharmacia Biotech Inc.) and then sequenced in the forward and reverse direction using the corresponding unlabelled V and JGT primer.

X chromosome inactivation patterns (XCIP)

Six of the cell strains derived from female patients were investigated for XCIP by genotyping using PCR primers for the human androgen receptor (HUMARA) (15). PCR was carried out as described above. The amplified products were electrophoresed on acrylamide gels using a 373A automated sequencer and analysed using Genescan 672 software (ABI).

Cell-mediated lymphocytotoxicity assay

After cell strains were established and if patients were available during follow-up visits for venipuncture, mononuclear cells were isolated from blood using standard Lymphoprep techniques. One-way mixed lymphocyte cultures (MLC) were set up by mixing 2.5×10^5 effector (E) peripheral blood lymphocytes with 2.5×10^5 irradiated target (T) autologous lymphocytes from the cell strain in complete tissue culture medium RPMI-1640. The target cells (T) received 3000 rad from a Cobalt 60 source (Atomic Energy of Canada Ltd). The cultures were set up in 96-well round bottom microtest plates in triplicates or quadruplicates in a total volume of 250 μl/well at 37°C in 5% CO_2. The generation of cytotoxic lymphocytes was evaluated after a 12-day MLC with a 4h ^{51}Cr release assay (Amersham), by adding 5×10^4 ^{51}Cr-labelled target (T) cells from the autologous T-cell strain to each well, thus giving an E:T ratio of 5:1. Allogenic MLC were used as positive control, and the patients own peripheral lymphocytes as negative control. Spontaneous release and maximum release were determined by adding target cells to wells containing either complete medium or 1% Triton X-100 for cell lysis, respectively. 100 μl supernatant was harvested from all wells and counted using a gamma counter (1272 Clinigamma, Turku, Finland). Cell-mediated lymphocytotoxicity assay was measured as "percentage specific lysis" = (experimental cpm − spontaneous cpm) $\times 100/$(maximal cpm − spontaneous cpm) (16).

Statistical analysis

The Wilcoxon rank sum test was used for statistical analysis.

RESULTS

A total of 27 skin biopsies were taken from 24 patients. Only one became infected and was thus discarded (no clinical signs of skin infection). Three skin biopsies were taken from individual skin tumours of patient # 164 (Table I) and all gave rise to individual cell strains, demonstrating almost 100% efficacy in establishing cell strains.

Outgrowth of cells

Lymphocyte expansion began within 2 weeks and averaged $62 \pm 30 \times 10^6$ after 4 weeks and $134 \pm 70 \times 10^6$ after 8 weeks. The highest number of lymphocytes was 495×10^6 cells at 8 weeks of culture from a patient with clinically very active and widespread CTCL. The cell strains were viable for up to 13 weeks, but none showed continuous growth. Proliferation was strongest during the first 2 months, after which growth was on a plateau suggesting senescence or steady state in which the number of live cells is kept constant through proliferation and disappearance of cells. All investigations were done within the 8 week growth period, where the total cell number was increasing.

We took 2 cell strains exhibiting strong growth and seeded them at 240,000, 24,000, 2400, 240, 120 and 60 cells into 5 ml of medium. Two weeks later we observed 3.7×10^6 from 240,000 cells, 3.0×10^6 from 24,000 cells, and 0.3×10^6 lymphocytes from 2400 cells, respectively. No significant growth occurred in flasks below 2400

cells (i.e. 580 cells/ml), indicating the need for a certain cellular density to achieve growth of lymphocytes. Nine different T-cell strains were taken and 0.1×10^6 cells seeded into culture flasks with 5 ml of medium (20,000 cells/ml). Following 9 days of growth we had a total of $2.4–13.5 \times 10^6$ lymphocytes, which would correspond to a cellular doubling time of between 30 and 43 h.

Morphology of the cell strains

Fig. 1 shows how the cells appeared in haematoxylin staining and by scanning electron microscopy. Most cells were lymphoblasts, some showed large, indented nuclei. Fig. 2 shows a laser confocal microscopic picture of a multi-lobulated nucleus indicating prominent changes in ploidy, e.g. DNA packaging. The cells shown in Figs 1 and 2 are from the same patient with fatal T-cell lymphoma (# 438, Table I).
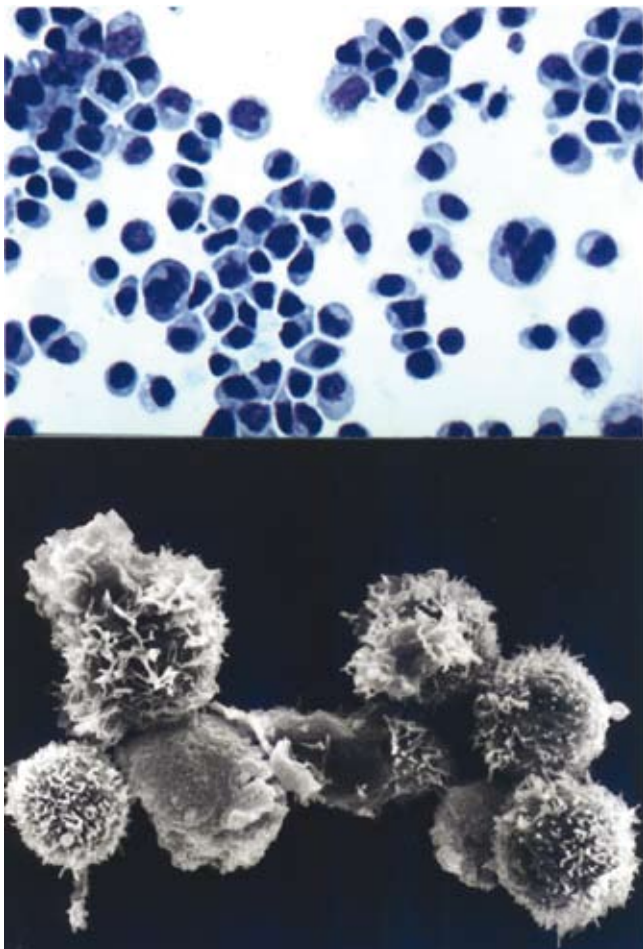

Fig. 2. Laser confocal pictures from the cell strain of patient 438 with a fatal T-cell lymphoma. Significant lobulation of the nucleus is evident.

Cell cycles in CTCL cell strains

Cell cycle determinations were performed among 10 cell strains in strong proliferation before 8 weeks of culture. A median of 28% (range 13–37%) of the cells were in S phase and 0–3% in apoptosis.

Phenotype analysis of cell strains

In some cell strains we observed up to 15% of lymphocytes, which did not stain for the TCR or CD3 (Fig. 3). Some of these cells were found to be natural killer (NK) cells as they stained for CD16+CD56+. In 3 cell lines we observed a decrease in T lymphocytes expressing α/β receptors and an increase in T lymphocytes expressing TCR-γ/δ (see Fig. 3). Staining for monocytes and B lymphocytes were negative (results not shown).

Over time a shift occurred in the CD4+:CD8+ ratio in most T-cell strains (Table I and Fig. 4). Thus, CD8+ cells became prominent in 17 of 24 cell strains with a ratio < 2, in contrast to 7 cell strains where CD4+ T cells were prominent (ratio >2) during *in vitro* culture ($p < 0.05$). Of these patients, 2 had parapsoriasis and they did not show an increase in CD4+ cells relative to CD8+ cells. Five patients had extensive and progressive disease and clearly developed prominent growth of CD4+ cells (Table I).

When looking at the semi-quantitative histology scoring of the skin biopsies and the outcome of *in vitro* growth, we observed that outgrowth of CD8+ T lymphocytes took place in cultures from patients who had the lowest score in their skin biopsy and also an early stage of the disease (Table II). This difference reached statistical significance ($p < 0.05$).

TCR gene re-arrangement

Twenty-one of 24 cell strains were available for analysis. Table III shows the number of clones measured in relation to the culture period. We could detect, on average, 12 T-cell clones after 2 weeks of culture. At 8 weeks of culture this was reduced to 6 T-cell clones, which fell to 5 clones after 12 weeks of culture. There was no difference between cell lines from parapsoriasis patients or patients with advanced disease.

Fig. 1. Haematoxylin-staining of a cell strain showing the cells are lymphoblasts (*top*). This cell strain was from patient 438 (Table I) who had fatal T-cell lymphoma. Scanning electron microscopy shows lymphoblasts (*bottom*) which morphologically are similar to phytohaemagglutinin-stimulated peripheral blood lymphoblasts (not shown), but did not reveal changes specific for cutaneous T-cell lymphoma cell strains compared with normal blood lymphoblasts.
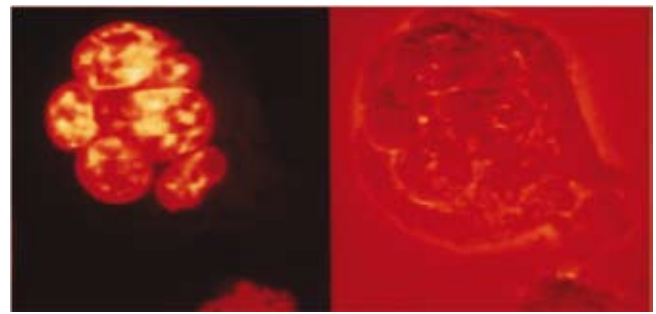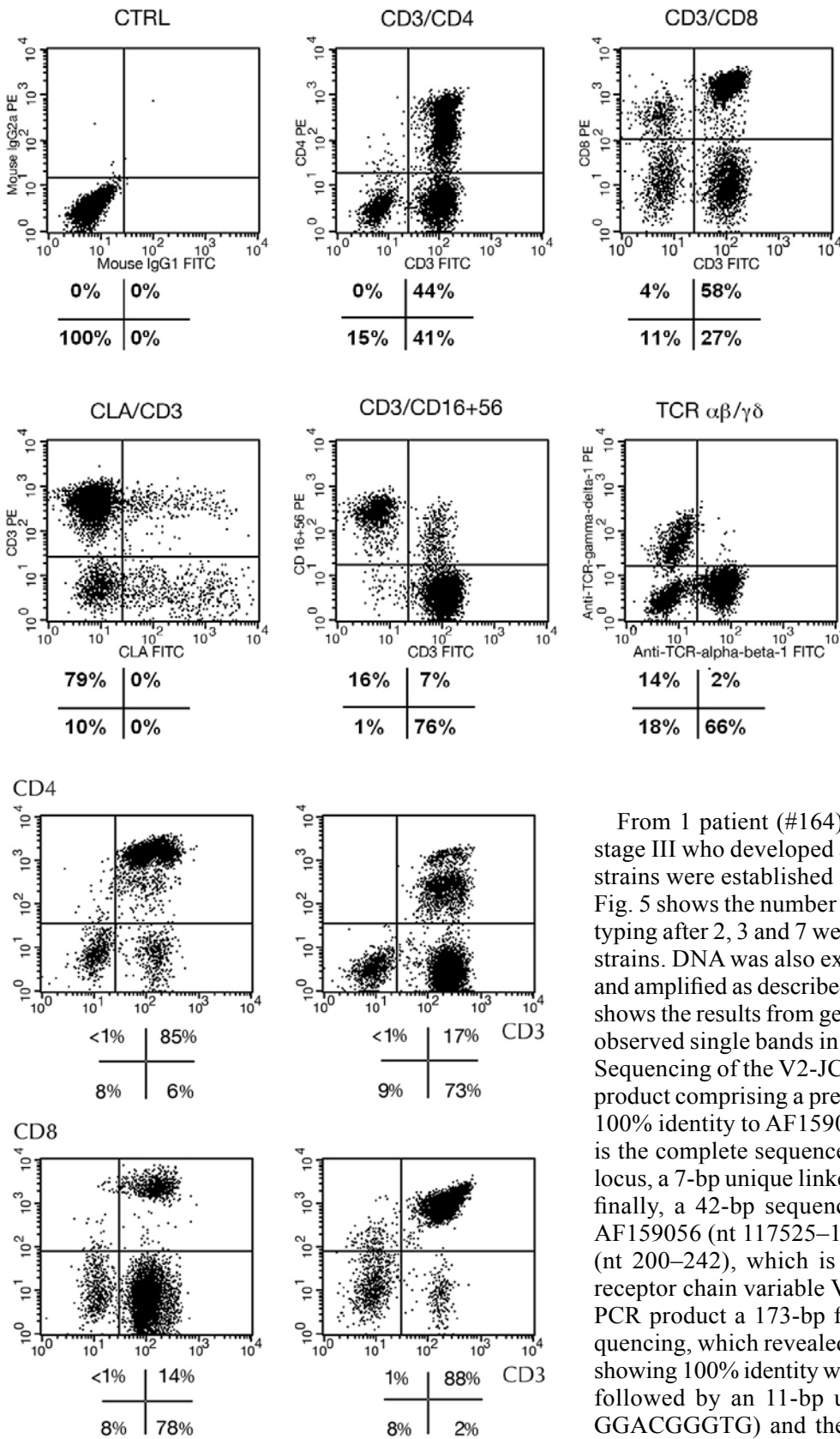
Fig. 3. FACS phenotypic analysis of cell strain #164 after 3 weeks of culture. The figures should be read from the upper left panel going right and then from the lower left panel. Above each individual figure is the studied phenotype e.g. first figure is CTRL which is the control experiment with non-specific isotype antibodies. The antibodies used are indicated on the abscissa and the ordinate of each individual figure and below are the percentages of cells found in each of the four squares. As examples the 2nd and the 3rd figures in the upper part of the panel shows approx. 15 to 11% of the cells did not stain for the CD3 marker. This is confirmed in the lower panel in the figure in the middle figure (CD3/CD16+56), where around 16% of the cells were negative for CD3, but positive for CD16+56 meaning they are natural killer (NK) cells. Fourteen percent of the T cells expressed TCR-γ-δ receptors

Fig. 4. FACS phenotype analysis of a cell strain over culture time demonstrating the change from a predominant CD4+ phenotype to a predominant CD8+ phenotype. The left column shows the results after 2 weeks of culture, the right column shows the results after 4 weeks of culture.

From 1 patient (#164), a 42-year-old man with MF stage III who developed a number of skin tumours, cell strains were established from 3 different skin tumours. Fig. 5 shows the number of clones determined by genotyping after 2, 3 and 7 weeks of culture of one of the cell strains. DNA was also extracted from 3 of the biopsies, and amplified as described in the Methods section. Fig. 6 shows the results from genotyping of the skin tissue. We observed single bands in 5 of 6 possible PCR reactions. Sequencing of the V2-JCT 3 product revealed a 177-bp product comprising a preceding 128-bp region showing 100% identity to AF159056 (nt 10975 – 11087), which is the complete sequence of the human T-cell receptor locus, a 7-bp unique linker sequence (GGTCGTG) and, finally, a 42-bp sequence showing 100% identity to AF159056 (nt 117525–117567) and also gbl S60175.1 (nt 200–242), which is the TCR V3-JP2-C 2+T-cell receptor chain variable V-J junction. For the V4-JCT 3 PCR product a 173-bp fragment was obtained for sequencing, which revealed a preceding 126-bp sequence showing 100% identity with AF59056 (nt 20130–20234) followed by an 11-bp unique linker sequence (AGGGACGGGTG) and then a 36-bp sequence showing 100% identity to AF159056 (nt 117525–117562). The remaining PCR products (V4-JCT 12 and V4-JCT 12) did not reveal unique linker sequences although 100% identity to AF159056 was observed (nt 10967–11088

Table III. *Number of T-cell clones in cell strains during in vitro culture*

	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7	Week 8	Week 12
Median number of clones	12	11	10	7	8	7	6	5
Range of clones	7–22	6–16	5–14	4–10	6–13	3–11	3–9	2–8
Number of cell strains studied	5	6	12	6	6	6	4	5

The table shows how many T-cell clones were found during *in vitro* culture of the cell strains, as determined by genotyping of the T-cell receptor locus.

and 20124–20238, respectively). Nor was there any match for V4-JCT 4. Both specific clones were present in the cell strain after 2 weeks of culture, but not at later time points, suggesting that these unique clones had been eliminated from the cell strain.

Cytotoxicity studies

We were able to perform cell-mediated lymphocytotoxicity assay in 5 patients and the results are shown in Table IV. All patients had peripheral blood lymphocytes, that could develop a specific cytotoxic response towards X-irradiated autologous skin-homing T cells. In one patient where 3 cell strains were established (#164), his peripheral blood lymphocytes were cytotoxic to all cell strains.

X chromosome inactivation studies

We observed that 3 of our 7 CTCL cell lines from women were homozygous for the HUMARA gene, which left 4 heterozygous cell lines for evaluation. We did not observe XCIP, indicating that the malignant T-lymphocytes are not derived from a single stem cell.

DISCUSSION

Cell strains can be established from skin biopsies of patients with inflammatory skin diseases and thus are not restricted to CTCL (17). We previously observed an average CD4+:CD8+ ratio after 8 weeks of *in vitro* growth of 1.20 (atopic dermatitis $n=19$) and 0.85 (psoriasis, $n=9$) (17). In the present study the CD4+: CD8+ ratio for early CTCL was initially 2.72, which dropped to 0.33 ($p<0.05$) and for progressive CTCL an increase was observed from 5.51 to 24.0 (Table I).

We have previously used this *in vitro* technique to analyse karyotypes of skin-homing T lymphocytes from CTCL patients. We observed that many cells in the cell strains underwent quite dramatic changes, including deletions, translocations and extra chromosomes (18, 19), although no uniform pattern was observed, as confirmed by others (20). These findings indicate that many cells are destined for malignancy, as genetic instability can be regarded as a parameter for predisposition to cancer (21). Recently, it was found that neoplastic T cells in CTCL bear both a dominant TCR rearrangement and a complex chromosomal aberration (22). Further evidence for the presence of malignant T cells in our cell strains
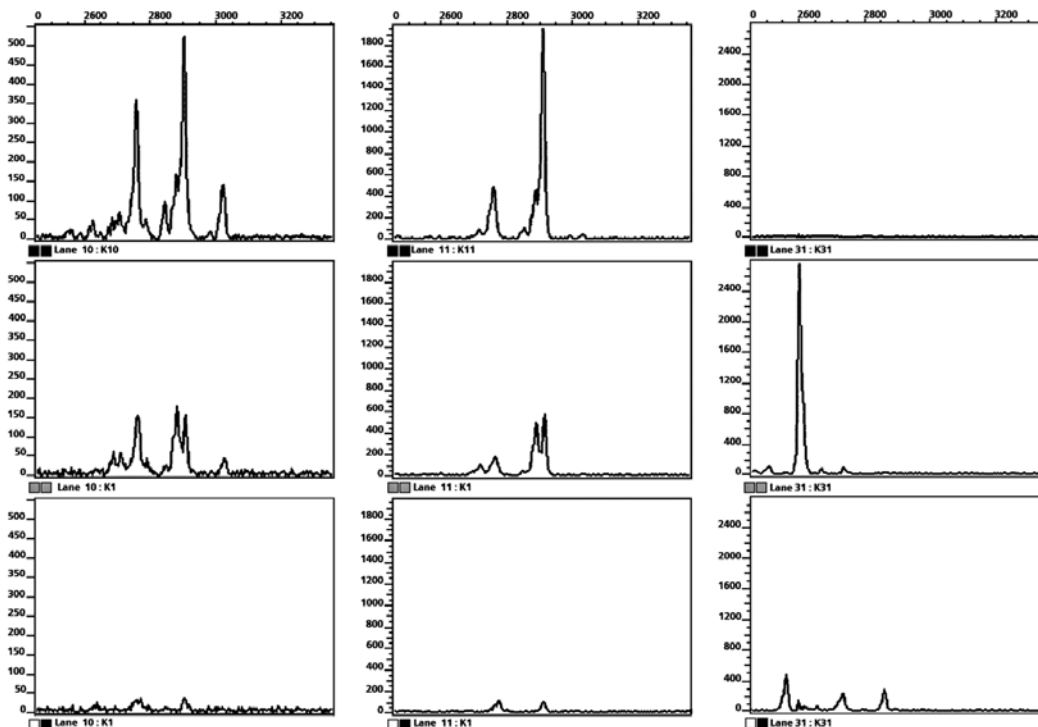

Fig. 5. Gelelectrophoresis of amplified regions of the TCR-α gene. A cell strain from patient #164 analysed for T-cell clones after 2, 3 and 7 weeks of culture (left, middle and right column, respectively) with primers for V2, V4 and V3 (upper, middle and lower row, respectively). It is clearly seen how some clones disappear (see upper row), whereas other become prominent during the culture period (see middle row, right column).
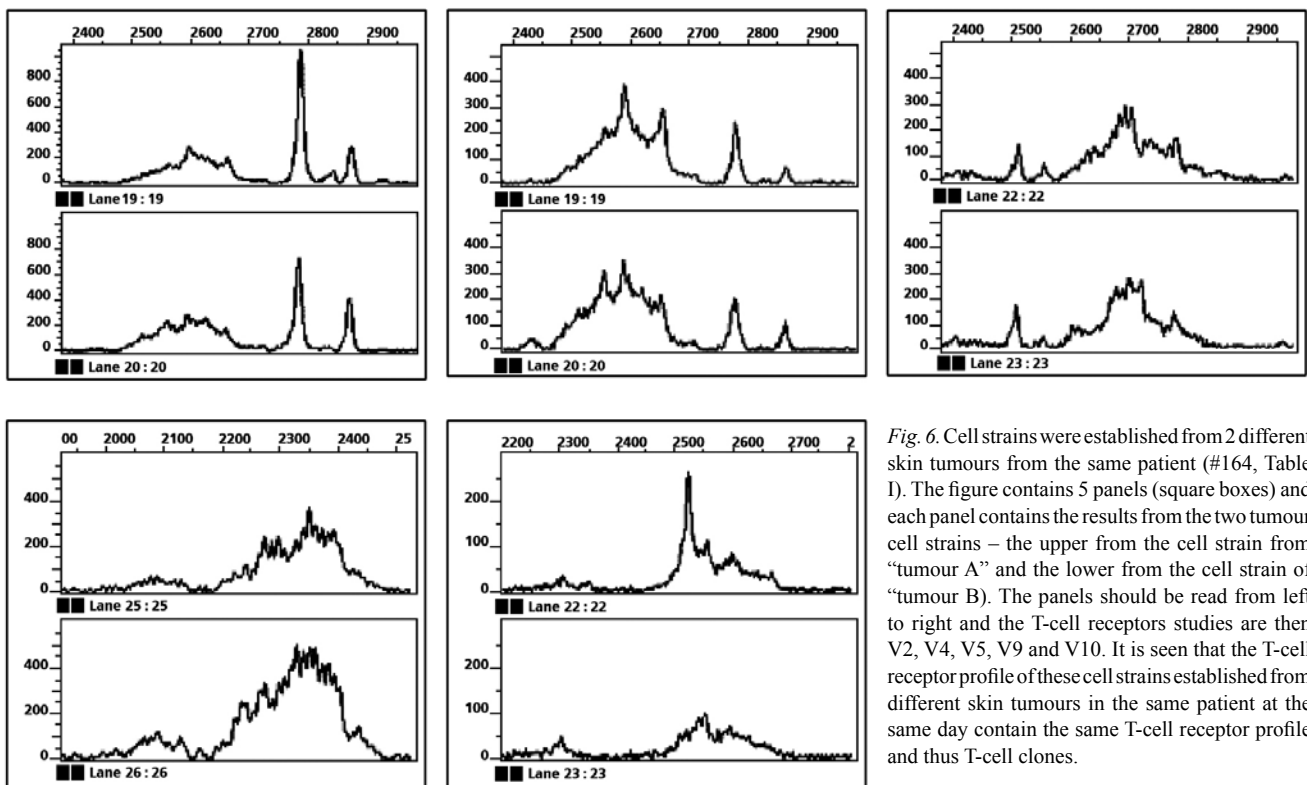
Fig. 6. Cell strains were established from 2 different skin tumours from the same patient (#164, Table I). The figure contains 5 panels (square boxes) and each panel contains the results from the two tumour cell strains – the upper from the cell strain from "tumour A" and the lower from the cell strain of "tumour B". The panels should be read from left to right and the T-cell receptors studies are then V2, V4, V5, V9 and V10. It is seen that the T-cell receptor profile of these cell strains established from different skin tumours in the same patient at the same day contain the same T-cell receptor profile and thus T-cell clones.

is the expression in the cell strains of cTAGE gene and protein (23).

Although most lymphocytes were T cells, certain cell strains displayed the presence of 10–15% of cells, which did not express CD3 or T-cell receptors (Fig. 3). Some of these cells were NK cells (CD16+56+). NK cells were infrequent early in the cultures (from 0% to 8%; $n = 6$, 2nd week of culture), but increased later in some cultures up to 30%. Three cell strains developed a dominance of T lymphocytes expressing γ/δ T-cell receptors and not the usually observed α/β TCRs. The change in CD4+:

Table IV. *Percentage of specific cytotoxicity as measured by* ^{51}Cr *release assay*

MR #	Effector cells (E) alone (%)	Target cells (T) alone (%)	E+T-cells (%)	Non-MHC-related target cells (%)
1	0	−10	32	na
41	−2	−3	9	−1
295	4	0	6	na
164A	4	1	19	4
164B	5	3	24	6
164C	−7	−8	24	−1
433	4	−3	18	na

MR #, medical record number; MHC, major histocompatibility complex; na, not available.
Non-MHC-related target cells were cells from an allogeneic donor, which had not been pre-incubated with effector cells, thus demonstrating that the killing was specific for autologous skin-homing T-cells and was MHC-specific. Percentage of specific killing was calculated according to the following formula: (experimental cpm − spontaneous cpm) ×100 / (maximal cpm − spontaneous cpm).

CD8+ ratio (Table II and Fig. 4) again demonstrates the dynamics of the *in vitro* cultures, where a group of CD8+ cells becomes dominant, most likely via cytotoxic activity towards CD4+ T cells (Table IV).

Proliferation is polyclonal or oligoclonal, with on average 12 clones being reduced to 5 over a 3-month culture period (Table III). There seemed to be no correlation with the clinical stage of the disease. Recently, a study on skin, blood and lymph node samples from patients with CTCL showed similar findings of clones using the GeneScan technique (24). Our *in vitro* observations are in full accordance with the observations *in vivo* of a profound loss of T-cell receptor repertoire complexity (25).

Although our cell-mediated lymphocytotoxicity studies are preliminary, they indicate that lymphocytes from blood could develop specific cytotoxicity towards cells among the skin-homing T lymphocytes. This is in line with the clinical observation that the presence of CD8+ T cells in patients with MF influences the long-term survival of the patients (7, 8, 26). A recent study confirmed that the number of CD8+ T cells present in skin biopsies correlated significantly with early-stage CTCL (27). This is further supported by the observation that CTCL patients with malignancy of CD8+ T cells have a poor prognosis (28). IL 12 therapy of CTCL patients shows an increase in CD8+ T cells in regressing lesions (29).

Our observation of the development of CD8+ T cells among the skin-homing cell strains is supported by a

recent observation of tumour-specific cytotoxic CD4+ and CD4+CD8dim+ T-cell clones infiltrating a cutaneous T-cell lymphoma (30). It is also known that only patients who still have a significant number of CD8+ T lymphocytes in peripheral blood will benefit from extracorporal photophoresis (31). Thus, the presence of CD8+ T cells *in vivo* (27) and, as demonstrated in this study, *in vitro,* is significantly associated with a clinically benign course of the disease. It could be argued that predominant growth *in vitro* of CD8+ T-cells is due to lack of growth of CD4+ T cells in the culture – including malignant CD4+ T cells. However, this cannot be the case, because, as seen in Table I, patients with clinically advanced CTCL have cell strains with a higher number of CD4+ cells than CD8+ cells. These advanced-stage patients have a high number of malignant T cells in their skin. The patient (#295) with the highest cumulative number of cells (495 million) over the 8-week growth period had a CD4+:CD8+ ratio at the final measurement of 24.0, demonstrating that CD4+ can show strong growth *in vitro*.

The X chromosome inactivation studies seem to exclude the idea that malignant T lymphocytes derive from a unique stem cell. Rather, the malignant transformation can take place in multiple mature T lymphocytes. Also, it was observed recently that 59% of CTCL patients have a novel splice variant of the *Fas* gene leading to impaired Fas-induced apoptosis, a change taking place in many different T lymphocytes and a potential contributing factor for an increased growth potential of T lymphocytes (31, 32).

Our model allows for further analysis of the complex interactions taking place in the skin of patients with CTCL by using an *in vitro* method to expand the skin-homing T lymphocytes. The observation of a cytotoxic response of blood lymphocytes towards autologous skin-homing T-cell strains requires further study.

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