Dominance of Nonmalignant T-Cell Clones and Distortion of the TCR Repertoire in the Peripheral Blood of Patients with Cutaneous CD30$^+$ Lymphoproliferative Disorders

Daniel Humme[1], Ansgar Lukowsky[1], Matthias Steinhoff[1], Marc Beyer[1], Peter Walden[1], Wolfram Sterry[1] and Chalid Assaf[1]

The CD30-positive cutaneous lymphoproliferative disorders (CLPD) include lymphomatoid papulosis (LyP) and primary cutaneous anaplastic large T-cell lymphoma (cALCL). Despite the malign-appearing histology, an excellent prognosis and spontaneous regression of single lesions characterize LyP. Even after years of clinical remission newly erupting lesions often harbor a T-cell clone identical to the initial one. This fact raises the question whether the clonal T-cell population persists in the peripheral blood. Therefore we investigated genomic DNA of 126 samples of lesional skin and peripheral blood from 31 patients with CLPD, obtained during both active disease and clinical remission. We performed molecular genetic analysis by combining T-cell receptor (TCR)-γ PCR with the GeneScan technique and assessed the TCR repertoire in selected blood samples by β-variable complementarity-determining region 3 (CDR3) spectratyping qualitatively and quantitatively. We were able to detect a clonal T-cell population in 36/43 (84%) skin samples and in 35/83 (42%) blood samples. Comparison of the compartments in each patient demonstrated different T-cell clones in skin and blood, suggesting a reactive nature of the clonal T cells in the blood. Moreover, CDR3 spectratyping revealed a restricted T-cell repertoire in the blood, suggesting T-cell stimulation by an unknown antigen.

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INTRODUCTION

Primary cutaneous T-cell lymphomas are a heterogeneous group of neoplasms, originating from clonal T cells. Apart from mycosis fungoides (Mf), primary cutaneous CD30$^+$ lymphoproliferations (CLPD) are the most common entities, accounting for approximately 30% of all cutaneous T-cell lymphomas. This group includes lymphomatoid papulosis (LyP) and primary cutaneous anaplastic large T-cell lymphoma (cALCL), and represents a range of disease with variable morphology, immunophenotype, and clinical behavior (Macaulay, 1968; Willemze *et al*., 2005; Droc *et al*., 2007).

Macaulay (1968) first described LyP as a chronic disorder, which is characterized by recurrent self-healing dome-shaped papules often spreading over a period of months or several years. The disease usually occurs in adults with a median age of 45 years (male to female patient ratio: 1.5:1) but may occur in children as well (Willemze *et al*., 2005). The lesions typically involve the trunk and extremities and may be in various stages of evolution. Patients with LyP have a benign clinical course with a 10-year survival rate of nearly 100% (Vergier *et al*., 1898; Macaulay, 1968; Paulli *et al*., 1995). However, due to an unknown reason, in 10–20% of the patients LyP can precede, coexist with, or follow malignant lymphomas, especially Mf, Hodgkin's lymphoma HL or ALCL (Kaudewitz *et al*., 1990; Davis *et al*., 1992). cALCL occurs predominantly in elderly adults, whereas occurrence in children is rare (Beljaards *et al*., 1993).

By using molecular biological techniques, for example, T-cell receptor (*TCR*) gene rearrangement analysis, clonal T cells could be identified in LyP (Weiss *et al*., 1986; Davis *et al*., 1992; Steinhoff *et al*., 2002; Gellrich *et al*., 2004). Previous studies have demonstrated that the identical T-cell clone could be detected in separate skin lesions and in skin biopsies taken at different time points (Chott *et al*., 1996; Steinhoff *et al*., 2002). Even in lesions recurring after several years of clinical remission the identical *TCR* gene rearrangement as in the initial lesions could be detected (Chott *et al*., 1996; Steinhoff *et al*., 2002). Moreover, identical clonal *TCR* gene rearrangements were also found in associated lymphomas

[1]*Department of Dermatology, Venerology and Allergy, Skin Cancer Center Charité, Charité – Universitätsmedizin Berlin, Berlin, Germany*

Correspondence: Dr. Chalid Assaf, Department of Dermatology, Venerology and Allergy, Skin Cancer Center Charité, Charité – Universitätsmedizin Berlin, Fabeckstr. 60-62, Berlin 14195, Germany. E-mail: chalid.assaf@charite.de

Abbreviations: cALCL, cutaneous anaplastic large T-cell lymphoma; CLPD, cutaneous lymphoproliferative disorder; LyP, lymphomatoid papulosis; Mf, mycosis fungoides; PBMC, peripheral blood mononuclear cell; sALCL, systemic anaplastic large T-cell lymphoma; TCR, T-cell receptor

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as Mf, ALCL, and Hodgkin's lymphoma (Kaudewitz *et al*., 1990, 1991; Wood *et al*., 1995; Chott *et al*., 1996), suggesting that LyP may be a precursor (Kaudewitz *et al*., 1990; Davis *et al*., 1992; Wood *et al*., 1995; Chott *et al*., 1996).

The fact that patients with LyP experience a relapsing course of their disease and additionally develop in 10–20% associated lymphomas even after years of clinical remission (Kaudewitz *et al*., 1991; Wood *et al*., 1995; Chott *et al*., 1996) raises the question of persistence of long-lived clonal T cells during the symptom-free period. Recently, Schultz *et al*. (2005) reported the detection of identical TCR rearrangements in skin and peripheral blood in 2/6 investigated patients. Interestingly, in 1/6 patients the T-cell clone could be detected in the peripheral blood during the symptom-free period. In contrast Whittaker *et al*. (1991) could not demonstrate a clonal T-cell population in the blood in 7/8 cases. However, there is a lack of broad systemic analyses to answer definitely the question whether the T-cell clone in the skin lesions also occurs in the peripheral blood.

In this study we first investigated if an identical T-cell clone in both skin and peripheral blood of a larger number of patients can be detected. Further on we analyzed the TCR-β repertoire qualitatively and quantitatively to determine whether an association between TCR clonality and TCR repertoire disturbances exists. To this end we analyzed and compared clonal *TCR-γ* rearrangements of 43 lesional skin biopsies and 83 peripheral blood mononuclear cell (PBMC) samples of 31 patients.

Skin and blood samples were taken and investigated at the times of active disease. Additional blood samples were investigated for clonality during the time of clinical remission. We applied the recently developed standardized Biomed 2 protocol in combination with the GeneScan analysis for *TCR-γ* rearrangements (van Dongen *et al*., 2003). Moreover, evaluation of the TCR repertoire of the peripheral blood of patients with CLPD was performed on mRNA level by T-cell spectratyping of the complementarity-determining region 3 (CDR3).

RESULTS

Analytical sensitivity of the TCR-γ Biomed 2 protocol

The analytical sensitivity of the TCR-γ Biomed II PCR in combination with the fluorescence fragment analysis technique was determined by serial dilution of the T-cell line MyLa in pooled PBMCs of 10 healthy donors. The PCR assays had a detection threshold of 2–5% clonally rearranged DNA in a polyclonal background (Figure 1).

Detection of a clonal T-cell population in skin lesions of CLPD

To determine the occurrence of a clonal T-cell population in lesional skin of patients with LyP and cALCL (patient characteristics are described in Table 1), we performed TCR-γ clonality analyses of genomic DNA of lesional T lymphocytes (Table 2). A clonal T-cell population could be detected in lesional skin biopsies of 17/21 (81%) patients (21/27 biopsies (78%)) with LyP. In addition, multiple skin (2–3) specimens were available from four patients (patients 8, 9, 15, 19). In three of these patients an identical *TCR* gene rearrangement could be detected over a period of up to 3 years (representative example patient 9; Figure 2), demonstrating the persistence of the skin T-cell clone in LyP. A monoclonal T-cell expansion was detected in 6/7 (86%) patients with cALCL (11/12 biopsies, 92%), and in all three investigated cases with cutaneous manifestations of systemic anaplastic large T-cell lymphoma (sALCL), that is, in 4/4 biopsies (100%).

Detection of clonal T-cell population in the peripheral blood of CLPD

The phenomenon of spontaneous regression and recurrence of skin lesions observed in patients with LyP and cALCL raises the question of persistence of the clonal T-cell population in the peripheral blood. To determine clonality in the peripheral blood we investigated genomic DNA of 83 blood specimens for *TCR-γ* gene rearrangements (Table 2). Of the 31 patients 29 had at least one skin lesion at the time when blood was taken. The remaining two patients were in a state of clinical remission. In the majority of the patients blood samples were investigated during the clinical follow-up.

Clonal PCR products in PBMCs were detected in 8/21 (35%) patients with LyP (19/53 samples, 36%). In the other patients a polyclonal distribution of the analyzed PBMCs was found as exemplarily shown in Figure 3. Multiple (2–5) blood specimens were available from 14 patients and 6 of these patients demonstrated an identical clone in 2–4 separate samples. In these patients an identical T-cell clone was reproducibly detected over a period of at least 15 months as shown for patient 9 (Figure 2). Patients 1–4, 10, 15, and 18 of whom multiple blood samples were available consistently showed a polyclonal distribution. From five patients blood specimens were available during both active and inactive phases of their disease. An identical clonal T-cell population could be detected at both time points in three of these five patients (60%).

In cases of ALCL, a clonal *TCR* gene rearrangement could be detected in the peripheral blood in 6/7 patients (16/27 samples (59%) and in 0/3 patients with cALCL and sALCL, respectively. The investigation of multiple blood specimens was possible in 6/7 patients suffering from cALCL. Of the seven patients four had an identical clone in separate blood samples. In these patients the identical clone was demonstrated over periods of 14 months to more than 3 years.

In all, we were able to demonstrate clonal T cells in the skin samples of 26/31 (84%) patients with CLPD (17/21 (81%) LyP, 6/7 (86%) cALCL, 3/3 (100%) sALCL).

Moreover, we were also able to detect clonality in the peripheral blood in 14/31 (45%) patients diagnosed with CLPD (8/21 (38%) LyP, 6/7 (86%) cALCL, 0/3 (0%) sALCL).

However, direct comparison of the clonal T-cell population detected in the lesional skin and corresponding PBMC sample of each patient by fluorescence fragment analysis revealed a different rearrangement pattern in all instances.

The different clonal T-cell populations detected in the skin and corresponding blood samples could be followed up over years within the same patient (exemplary in Figure 2; all cases

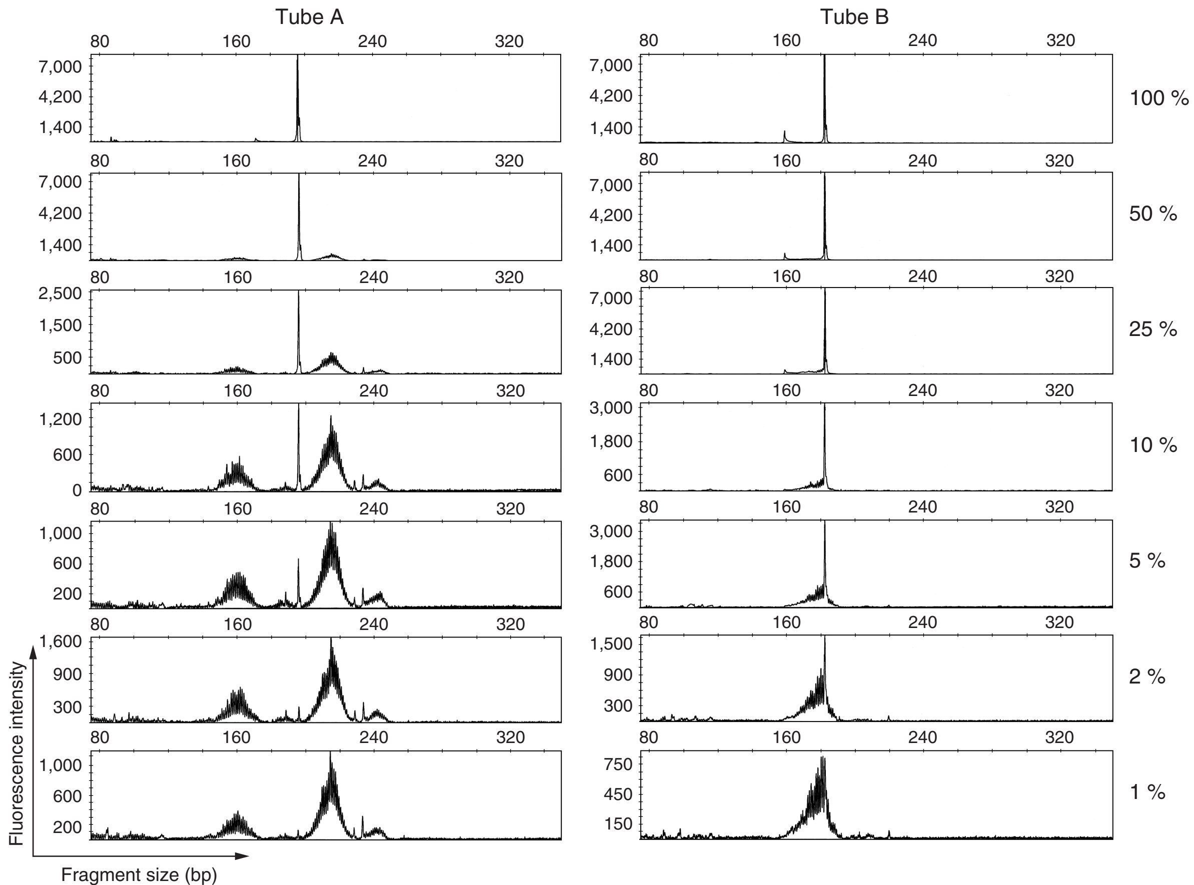

Figure 1. Serial dilution of the clonal T-cell line MyLa in pooled PBMCs of 10 healthy donors. *TCR*-γ PCR assays of tubes A (left side) and B (right side). Dilution steps were performed at 100, 50, 25, 10, 5, 2, and 1% clonal cells, showing a sensitivity of 2–5% (MyLa cell line reveals a PCR product of 196 base pairs in tube A and 182 base pairs in tube B); *x* axis, length of PCR products in base pairs (bp).

demonstrated in Supplementary Data). As controls, corresponding skin and blood samples of 10 patients with chronic dermatitis were analyzed. In none of these samples a monoclonal T-cell population was detected (data not shown).

Age distribution of patients with or without a clonal T-cell population in the peripheral blood

In previous studies it has been clearly demonstrated that there is a physiological age-related expansion of clonal T cells in PBMCs, after the age of 65 years (Posnett *et al.*, 1994; Schwab *et al.*, 1997; Muche *et al.*, 2003; Vallejo, 2007). Therefore we compared the average age of patients with clonal TCR-γ and a polyclonal distribution of the *TCR*-γ gene rearrangement in the peripheral blood, respectively.

For LyP patients showing polyclonal TCR-γ distributions had an average age of 38 years (range: 13–88 years, $n=13$), whereas patients who had a clonal *TCR* gene rearrangement in the peripheral blood had an average age of 52 years (range: 30–70 years, $n=8$). In the six patients with cALCL showing a clonal *TCR*-γ gene rearrangement an average age of 50 years (range: 23–77 years, $n=6$) was calculated. The one patient with cALCL who had a polyclonal distribution of the *TCR*-γ gene rearrangements in the PBMCs was 14 years of age.

These data show that a clonal T-cell population in the peripheral blood occurs much earlier in patients with CLPD than physiologically expected for healthy elderly individuals.

Restriction of the TCR repertoire in the peripheral blood of patients with CLPD measured by CDR3 size analysis of TCR-β chain transcripts

For further characterization of peripheral blood T cells of patients with CLPD we looked for changes in the diversity of the TCR repertoire. This was performed by CDR3 spectratyping, allowing visualization of the TCR repertoire as by the TCR-β CDR3 distribution, on a single graph. In contrast to the GeneScan technique the immunoscope analysis is performed on the basis of mRNA. In this study the quantitative and qualitative CDR3 distributions of the peripheral blood T cells in 10 patients was analyzed (patients 1, 2, 5, 8, 12, 14, 15, 22, 23, 24). Patient 1 demonstrated an oligoclonal; patients 2, 5, 12, 14, 15, and 22 polyclonal; and patients 8, 23, and 24 monoclonal profile.

At the quantitative level, Vβ family transcripts were found accumulated in seven samples (Vβ2 in the sample of patient 8 and, to a lesser extent, in patients 5, 14, and 15; Vβ5.1 in patients 14, 15, and 24; Vβ6.1 in patient 1; Vβ6.5 in patient

Table 1. Clinical characteristics of the patients with CD30$^+$ lymphoproliferations

Patient	dx	Sex	Age at dx	Extent of skin lesion	Treatment	Course	Follow-up month	Current status
1	LyP, type A	M	60	Multifocal	Excision, local steroids, MTX	SD	32	A w d
2	LyP, type A	M	13	Multifocal	PUVA, local steroid	SD.	26	A w d
3	LyP, type A	F	33	Localized	Excision, local steroids	SD	24	A w d
4	LyP, type A	M	52	Multifocal	Excision, MTX	CR	168	A w d
5	LyP, type A	F	31	Multifocal	PUVA, MTX	SD	73	A w d
6	LyP, type A	M	40	Multifocal	PUVA, systemic retinoids, MTX	CR	96	A w/o d
7	LyP, type A	F	38	Localized	PUVA, excision	SD	13	A w d
8	LyP, type A	M	56	Localized	Excision	SD	55	A w d
9	LyP, type A	M	64	Multifocal	MTX, PUVA, local steroids	SD	36	A w d
10	LyP, type A	F	54	Solitary	Excision, MTX	CR	120	A w/o d
11	LyP, type A	M	81	Multifocal	Excision, radiation, chlorambucil, flucytoarabin	PD	27	DOD
12	LyP, type A	M	26	Multifocal	Observation	SD	12	A w d
13	LyP, type A	M	26	Localized	PUVA	CR	33	A w/o d
14	LyP, type A	F	24	Multifocal	MTX	CR	13	A w/o d
15	LyP, type A	F	17	Multifocal	Local steroids	CR	39	A w/o d
16	LyP, type A	F	58	Multifocal	MTX	SD	32	A w d
17	LyP, type A	F	49	Multifocal	PUVA, MTX, IFN-α	SD	15	A w d
18	LyP, type A	M	32	Multifocal	MTX	CR	55	A w/o d
19	LyP, type B	M	73	localized	excision, PUVA, MTX	SD	33	A w d
20	LyP, type C	M	52	Localized	PUVA, excision, MTX	SD	31	A w d
21	LyP, type C	M	17	Localized	Excision, PUVA, MTX	CR	8	A w/o d
22	cALCL	M	32	Solitary	PUVA, radiation, excision, MTX	CR	17	A w/o d
23	cALCL	M	61	Solitary	Excision, radiation, IFN-α, MTX, CHOP	PD	44	A w d
24	cALCL	F	51	Solitary	Excision, MTX	CR	45	A w/o d
25	cALCL	M	16	Multifocal	Excision, MTX	SD	48	A w d
26	cALCL	M	36	Localized	Excision, radiation, vaccination, MTX, IFN-α	PD	22	DOD
27	cALCL	M	75	Solitary	Excision, radiation	SD	30	A w d
28	cALCL	M	24	Solitary	Excision, radiation	CR	27	A w/o d
29	sALCL	M	37	Localized	CHOP, alemtuzumab	PD	27	A w d
30	sALCL	M	52	Localized	CHOP, radiation, MTX, doxorubicin, vepisid	PD	42	A w d
31	sALCL	M	18	Localized	BMT	CR	66	A w/o d

Abbreviations: A w d, alive with disease; A w/o d, alive without disease; BMT, bone marrow transplantation; cALCL, cutaneous anaplastic large cell lymphoma; CHOP, cyclophosphamide, hydroxydaunorubicin, oncovin (vincristin), prednisolone; CR, complete remission; DOD, died of disease; dx, diagnosis; F, female; IFN-α, interferon-α; LyP, lymphomatoide Papulose; M, male; MTX, methotrexate; PD, progressive disease; PUVA, psoralene with UVA-phototherapy; sALCL, systemic anaplastic large cell lymphoma; SD, stable disease.

5; Vβ13.1 in patients 2, 5, 14, 15; and Vβ17 in patient 24). On the other hand, other Vβ families (mainly the Vβ13.5, Vβ15, Vβ23, and Vβ24 families) were only weakly represented in most of the samples.

At the qualitative level, an altered CDR3 length distribution profile in comparison to a Gaussian distribution was observed for the majority of the Vβ families in patients 1, 8 and to a lesser extent, in patients 22 and 23. In the other samples most of the Vβ families displayed a Gaussian length distribution.

Comparison of the *TCR-γ* gene rearrangement analyses and the analyses of the CDR3 of TCR-β

The comparison of T-cell clonality and immunoscope analysis for patient 8 with LyP who had developed an associated lymphoma (Mf) presented a monoclonal *TCR-γ* rearrangement in the GeneScan and a very strong distortion of the CDR3 distributions. In contrast, patient 1 with LyP demonstrated an oligoclonal *TCR-γ* rearrangement had a less distorted CDR3 length distribution. Patient 15 with LyP showed a polyclonal TCR rearrangement profile (GeneScan)

Table 2. Patient and specimen information

Patient	Entity	No. of cases with clonality in skin	Total of investigated skin cases	Size in bp (skin)	No. of cases with clonality in blood	Total of investigated blood cases	Size in bp (blood)
1	LyP, type A	0	1	NA	0 (five oligo)	5	NA
2	LyP, type A	1	1	216, 252 (A)	0	5	NA
3	LyP, type A	1	1	114 (B)	0	3	NA
4	LyP, type A	0	1	NA	0	3	NA
5	LyP, type A	1	1	103 (B)	0	1	NA
6	LyP, type A	0	1	NA	2	5	155 (A)
7	LyP, type A	0	1	NA	1	2	107 (B)
8	LyP, type A	2	3	184,216 (A)	3	3	186 (B)
9	LyP, type A	3	3	145 (A)	3	3	159 (A) 112 (B)
10	LyP, type A	1	1	160,209 (A)	0	4	NA
11	LyP, type A	1	1	241 (A) 178 (B)	0 (one oligo)	1	NA
12	LyP, type A	1	1	218 (A) 184 (B)	0	1	NA
13	LyP, type A	1	1	177 (B)	4	4	174 (A)
14	LyP, type A	1	1	182 (B)	0	1	NA
15	LyP, type A	2	2	176 (A) 178 (B)	0	2	NA
16	LyP, type A	1	1	202,237 (A)	2	2	208 (B)
17	LyP, type A	1	1	215,229 (A)	1	1	150 (A)
18	LyP, type A	1	1	216,156 (A)	0	2	NA
19	LyP, type B	1	2	215 (A) 161 (B)	3	3	244 (A)
20	LyP, type C	1	1	216,219 (A)	0	1	NA
21	LyP, type C	1	1	154,236 (A)	0	1	NA
22	cALCL	0	1	NA	2	3	142 (B)
23	cALCL	3	3	157 (A)	2	5	152 (A)
24	cALCL	1	1	152,212 (A)	6	6	210,247 (A)
25	cALCL	1	1	215 (A) 180 (B)	0	1	NA
26	cALCL	2	2	155 (A) 194 (B)	1	5	218 (A) 110,117 (B)
27	cALCL	2	2	215 (A)[1]	4	4	243 (A) 157 (B) in 3/4
28	cALCL	2	2	151 (A)	1	3	159 (B)
29	sALCL	1	1	159 (A)	0 (one oligo)	1	NA
30	sALCL	2	2	173 (B)	0 (one oligo)	1	NA
31	sALCL	1	1	241 (A)	0	1	NA

Abbreviations: (A), rearrangement found in tube A; (B), rearrangement found in tube B; cALCL, cutaneous anaplastic large T-cell lymphoma; LyP, lymphomatoid papulosis; Mf, mycosis fungoides; oligo, oligoclonal rearrangement; +1 add. peak, one additional peak was found; NA, not applicable; sALCL, systemic ALCL.
[1]Identical clone in tube A (215 bp). Different clones in tube B, one at 100 bp the other at 161 bp.

and a nearly Gaussian CDR3 length distribution in the Immunoscope analyses of peripheral blood (Figure 4).

All in all, three of the four investigated blood samples representing a clonal *TCR-γ* rearrangement demonstrated a clear distortion of the CDR3 distribution (patients 8, 23, 24). Although demonstrating clonality in the peripheral blood, patient 22 showed a nearly normal CDR3 length distribution. All of the five patients 2, 5, 12, 14, and 15 with a polyclonal *TCR-γ* rearrangement had a balanced TCR-β repertoire. Patient 1 with an oligoclonal profile in the GeneScan analysis showed a distorted CDR3 distribution as previously described.

Taken together, we were able to demonstrate a striking association of the degree of clonality and the extent of CDR3 distortions found in the peripheral blood. Patients with a polyclonal *TCR* gene rearrangement demonstrate a Gaussian-like distribution of the TCR repertoire, patients with an oligoclonal *TCR* gene rearrangement an altered, and patients with a dominant T-cell clone a severely distorted TCR repertoire.

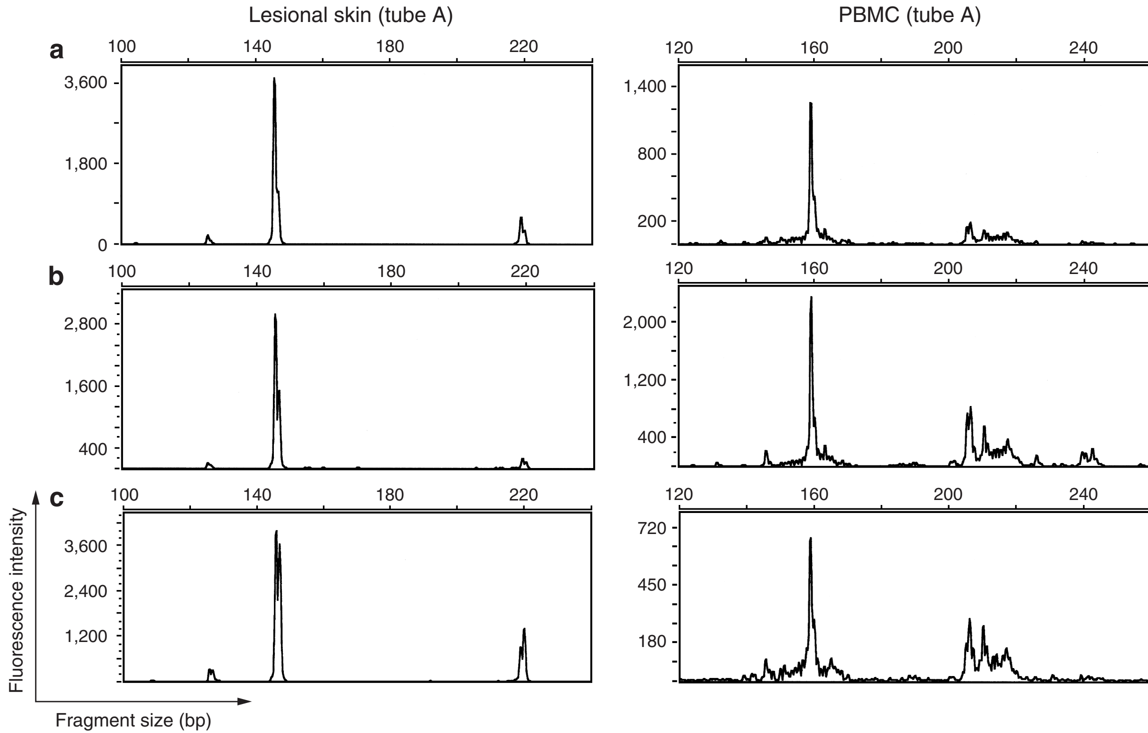

Figure 2. Comparison of clonal T-cell populations of lesional skin and peripheral blood of patient 9 (tube A). (Left side) Skin specimens were obtained in 01.1999 (**a**), 06.1999 (**b**), and 03.2002 (**c**) revealing an identical size of 145 bp. (Right side) Clonality of three separate blood specimens each with a fragment size of 159 bp. Specimens were obtained in 06.1999 (**a**), 03.2000 (**b**), and 03.2003 (**c**).

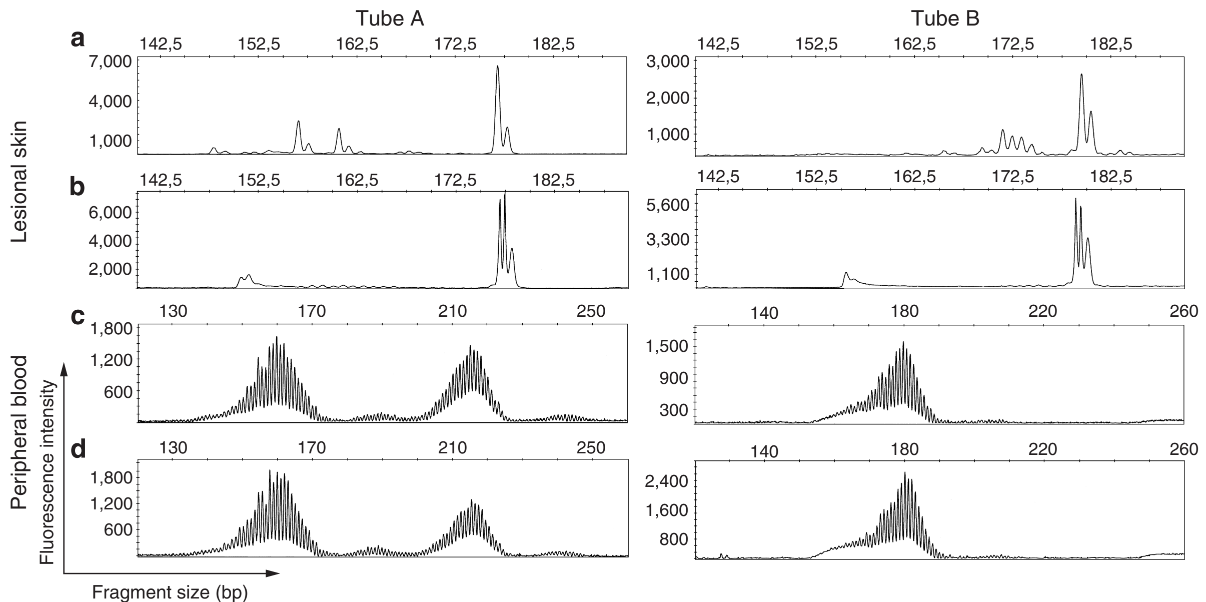

Figure 3. Comparison of clonal T-cell populations of lesional skin and peripheral blood. Identical clonal (biallelic) rearrangement of TCR -γ in two separate skin biopsies of the patient 15 with LyP (**a**, **b**). No clonal rearrangement (polyclonal distribution) in two separate blood samples (**c**, **d**) profiles. (Left side) Profiles of tube A, (right side) profiles of tube B.

DISCUSSION

Macauley (1968) called LyP a ''rhythmic paradoxical eruption'' and described it as a self-healing and usually recurrent papular disorder. At present it is unclear, in which compartments the clonal T cells persist during the relapse-free period of LyP. One hypothesis is that the neoplastic cells are disseminated in the peripheral blood and home back to the skin to cause new lesions. To answer the question if the

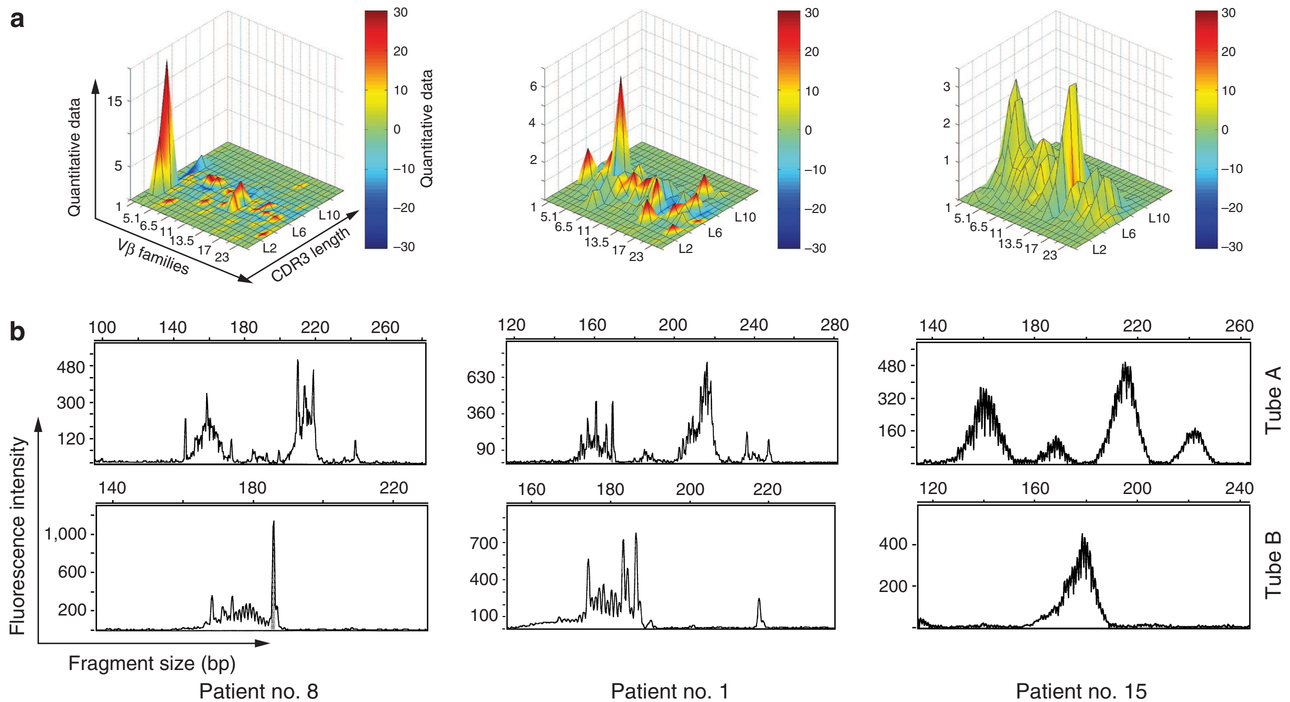

Figure 4. Correlation of immunoscope analysis with T-cell receptor gene rearrangement. Immunoscope analysis (**a**) and corresponding GeneScan profiles, tubes A and B (**b**). Patient 8 (left side) with LyP who developed an associated lymphoma (Mf) demonstrated a clear restriction in the T-cell receptor repertoire and a monoclonal *TCR-γ* rearrangement in the peripheral blood. Patient 1 (middle) with LyP demonstrated an oligoclonal *TCR-γ* rearrangement in association with an altered CDR3 length distribution whereas patient 15 (right side) with LyP presented a polyclonal GeneScan profile and a nearly Gaussian TCR-β CDR3 length distribution in the peripheral blood.

disease causing T-cell clone persists in the peripheral blood in CLPD, we performed a systematic analysis of 126 lesional skin and corresponding peripheral blood samples from 31 patients with special emphasis on LyP.

Our detection of a dominant T-cell clone in the skin of 81% of patients with LyP is in line with previous publications showing a range of 45–83% (Weiss *et al.*, 1986; Whittaker *et al.*, 1991; El Azhary *et al.*, 1994; Kadin, 2006). Our comparably high detection rate underlines the sensitivity of our approach. We also found a clonal T-cell population in skin biopsies of 86% of the patients with cALCL. This is in agreement with most studies showing a detectable T-cell clone in nearly all cALCL (Willemze, 2005; Kadin, 2006). In contrast to our findings, Greisser *et al.* (2006) detected clonality in none of nine investigated LyP type A cases, but in 50% of Lyp type C (4/8). The discrepancies between the published and our data reported in this study may be due to the different protocols for the analysis of TCR rearrangements. We used the sensitive Biomed primer sets which, according to extensive comparative studies, produce an increased detection rate of clonally expanded T cells in lymphomas with low numbers of tumor cells such as LyP type A.

In the peripheral blood we found a clonal T-cell population in 8/21 (38%) patients with LyP and in 6/7 (86%) patients with cALCL. However, intraindividual comparison of the clonal T-cell populations in blood and skin revealed different *TCR* gene rearrangements in all cases. Thus, within the sensitivity threshold of our technique of 2–5%, we were not able to detect an identical T-cell clone in the skin and the peripheral blood. So our results do not support the findings of Schultz *et al.* (2005). They may have overrated the detection of identical clones for the following two technical reasons. First, they used a primer set covering only approximately 75% of the possible *TCR-γ* gene rearrangements (McCarthy *et al.*, 1992). Second separation by PAGE, which only distinguishes PCR products with length difference of at least 10 base pairs (Scheller *et al.*, 1998) may wrongly give the impression of identical clones.

In case of patients with sALCL we could not identify clonal T-cell populations in the peripheral blood, which may be mainly due to the limited number of cases investigated.

Earlier, clonality analyses had been performed for other types of cutaneous T-cell lymphoma. (Muche *et al.*, 2003) detected identical *TCR* gene rearrangements in skin and peripheral blood, indicating a dissemination of the malignant T cells into the blood of patients with Mf. However, analogous to our data, subsequent studies on a large number of patients revealed distinct clonal proliferations in both compartments in 48–73% of the patients (Delfau-Larue *et al.*, 1998; Muche *et al.*, 2003). Moreover, clonal T-cell populations were detected in healthy individuals (5/38), in patients with autoimmune dermatoses (3/8), with Mf (8/64) and with cancer other than lymphoma (9/39) (Muche *et al.*, 2003).

As the finding of a dominant T-cell clone in the peripheral blood alone is not necessarily associated with a T-cell-dependent disease, we performed an immunoscope analysis

or CDR3 spectratyping of PBMCs. This technique provides more insight into the TCR repertoire by analyzing all 24 Vβ families separately. As the CDR3 spectratyping is performed on mRNA basis, quantitative aspects, for example, clonal activation as well as qualitative aspects like repertoire distortion, are taken into account. Our data from the immunoscope analysis demonstrate that the detection of a clonal TCR rearrangement in the peripheral blood is associated with a strong distortion of the CDR3 distribution and the TCR repertoire in most cases. In contrast, patients without a detectable T-cell clone in the blood showed a normal TCR repertoire. A pronounced overrepresentation of certain Vβ families at the quantitative level indicates a strong proliferation of subpopulations of T cells, maybe due to their antitumor immune response (Albers *et al.*, 2006). This hypothesis is supported by data published within the past years. For patients with Sézary syndrome the expansion of identical Vβ families in the skin and the peripheral blood were shown, representing identical T-cell clones (Ingen-Housz-Oro *et al.*, 2002). In addition to these dominant clones further expanded, clones varying with time were observed in the peripheral blood, suggesting nontumoral-reactive lymphocytes. Moreover, staining with CD 158k allowing to differentiate between malignant (CD 158k -positive) and nonmalignant (CD 158k -negative) T lymphocytes brought additional evidence for expanded reactive T-cell clones in patients with Sézary syndrome (Ortonne *et al.*, 2006).

In previous studies unrelated clonal T-cell proliferations in the peripheral blood as well as alterations in the TCR repertoire have been reported to occur frequently in patients over the age of 65 years (Posnett *et al.*, 1994; Schwab *et al.*, 1997; Muche *et al.*, 2003; Vallejo, 2007). Vallejo (2007) showed that the TCR diversity is usually maintained up to the age of 65 years and rapidly diminishes thereafter. However, patients from our study who demonstrated unrelated dominant T-cell clones in the peripheral blood were much younger than 65 years (average age 52 years) and all patients with distorted CDR3 distributions were under the age of 65 years (51, 59, 60, and 61 years).

Interestingly, distortions of the immune repertoire were also described in younger individuals suffering from chronic diseases such as CMV infections, which suggest that stimulation like persistent or periodic viral antigen spreading may have comparable effects on the T-cell repertoire (Looney *et al.*, 1999; Koch *et al.*, 2006). Restricted clonal dominance has been reported from immunoscope analyses of other forms of cutaneous lymphoma (Yawalkar *et al.*, 2003) and other cancers (Albers *et al.*, 2006) and interpreted by the authors as indicating contraction of the T-cell repertoires with substantial loss of TCR diversity. However, evaluating the clonality and immunoscope data in the context of the experiments assessing the sensitivity of the PCR-based analyses of TCR repertoires suggests an alternative interpretation. Already 10% monoclonal T cells admixed to a polyclonal T-cell population produce a clonality scan profile that mimics single clonal dominance in the test sample although still 90% of the cells are polyclonal. As in the immunoscope analyses the 24 TCR Vβ families are scanned separately, the sensitivity of detection of clonal dominance is here considerably enhanced. In addition, the qualitative Vβ distribution is similar to the findings of van den Beemd *et al.* (2000) in healthy individuals. Taken together, the earlier reports and our findings raise the possibility that the distortions of TCR repertoires and prominence of nonmalignant or else disease-associated T-cell clones in patients with CLPD may be related to chronic stimulation of the T cells by unknown antigens possibly associated with the CLPD.

In conclusion, we investigated clonal T-cell proliferations in the skin and the peripheral blood of 31 patients with CLPD. In addition, a clonal TCR rearrangement in a high percentage of skin samples our data demonstrate a different T-cell clone in the blood in 35 and 86% of patients with LyP and ALCL, respectively. Immunoscope analysis produced evidence that the clonal expansions detected in the peripheral blood are associated with an altered CDR3 length distribution pattern, which also occurred at a younger age and more frequently in patients with CLPD compared to reports for healthy individuals. We suggest that the unexpectedly high detection rate of clonality in the peripheral blood of patients with LyP might be due to a cancer-specific antigen-dependent T-cell proliferation. However, the exact nature of the skin-unrelated clonal T-cell proliferation in the blood remains to be resolved.

MATERIALS AND METHODS

Patients

The study included the analyses of genomic DNA from a total of 126 samples consisting of 83 blood and 43 skin samples from 31 clinically well-characterized patients with $CD30^+$ lymphoproliferations. All patients were treated and followed up by the Department of Dermatology, Charité, Berlin. This group consisted of 21 patients with LyP, 7 patients with cALCL, and 3 patients presenting with a secondary skin manifestation of sALCL. Detailed clinical information on all patients is summarized in Table 1. As a control skin and blood samples were investigated from 10 patients with chronic dermatitis.

According to the recent World Health Organization–European Organization for Research and Treatment of Cancer classification LyP was defined as recurrent nodular eruptions with evidence of spontaneous regression of skin lesions and histological features consistent with LyP. cALCL was defined as nodules or tumor lesions with histological features for cALCL and no evidence of extracutaneous manifestation at initial presentation (Willemze *et al.*, 2005).

The research committee of the Charité Universitätsmedizin Berlin has approved the described studies. Informed consent for the experimental studies was obtained from the patients. The study was conducted according to the Declaration of Helsinki Principles.

Characterization of specimens

All skin samples were examined by hematoxylin eosin staining and immunohistological staining (CD4, 1F6; Novocastra, Newcastle upon Tyne, UK; CD3, polyclonal CD3, CD8 (C8-144), CD45RO (OPD4), CD30 (Ber-H2), ALK (Dako, Glostrup, Denmark). To unmask the antigenic epitopes, the sections by high-pressure cooking in 10 mM citrate buffer (pH 6.0). According to the amount of $CD30^+$ atypical cells, LyP cases were subdivided into three

histological types designated as A with scattered CD30^{+} cells, B with absence of CD30^{+} cells, and C with sheets of CD30^{+} cells.

DNA preparation

For extraction of genomic DNA, 10 paraffin sections per sample (each 10 μm) were dissolved twice in 1 ml roticlear, centrifuged at 13,200 r.p.m. for 5 minutes and washed twice with ethanol followed by centrifugation as described earlier. To achieve a complete elimination of ethanol the specimens were dried in a dissicater. Extraction buffer (200 μl) and proteinase K (20 μl) (concentration 2.5 mg ml^{-1}, QIAamp DNA Kit; Qiagen; Hilden, Germany) were added and incubated overnight at 55 °C followed by 20 minutes at 95 °C and centrifugation at 13,200 r.p.m. for 10 minutes.

Genomic DNA from blood containing about 1×10^6 PBMCs was used. PBMCs were prepared from 10 ml heparinized blood per sample using Ficoll-HyPaque (Pharmacia, Freiburg, Germany). The DNA extraction was performed according to a standard procedure (Sambrook *et al.*, 1989). Genomic DNA from the MyLa cell line was extracted with a DNA purification kit (Blood DNA Purification Midi Kit; Qiagen) according to the manufacturer's instructions using the midi spin protocol for whole blood. The DNA concentrations were measured by absorbance at 260 nm.

TCR-γ PCR and fluorescent fragment analysis

TCR-γ rearrangements were amplified by PCR according to the standardized Biomed 2 protocol (van Dongen *et al.*, 2003). The cell lines MyLa and SeAx served as positive controls. Samples without DNA were used as negative controls in each assay. To confirm a successful amplification, 8 μl of each PCR product was screened on a 2% ethidium bromide-stained agarose gel for the detection of clonal bands or polyclonal smears.

For GeneScan analyses Jαp1/2 and Jγ1/2 Biomed 2 primers were fluorescence-labeled and used for PCR as described in the Biomed 2 protocol (van Dongen *et al.*, 2003). Fluorescence-labeled PCR amplificate (1 μl of each) was added to a mixture of 12.5 μl deionized formamide and 0.5 μl GeneScan 500TM Rox internal lane standard (PE Applied Biosystem, Weiterstadt, Germany). To produce single-stranded DNA, the amplificates were denatured at 95 °C for 5 minutes and chilled on ice. Following this procedure, the products were applied for GeneScan analyses to the ABI 310 PRISM CE sequencer (PE Applied Biosystem). Each run was performed under the following conditions: 60 °C, 15 kV, injection time of 5 seconds, separation time of 36 minutes, POP 6-filled 47-cm long capillary (PE Applied Biosystem). The data from each run were analyzed using the GeneScan 372 software (PE Applied Biosystem) Lukowsky *et al.*, 2003).

Determination of the sensitivity of the GeneScan analyses

The evaluation of the sensitivity of the GeneScan analyses was performed by serial dilution of DNA from a monoclonal T-cell line with DNA of polyclonal PBMCs. The polyclonal PCR products were generated from PBMCs of 10 healthy individuals. DNA was prepared with a DNA purification kit (Blood DNA Purification Midi Kit; Qiagen) and analyzed as above. For serial dilution, DNA of the MyLa cell line was mixed with the PBMC DNA at 50, 25, 10, 5, 2, and 1%. DNA (5 μl) with 50 ng DNA per μl was used per PCR for fluorescence fragment analysis. Two independent assays were performed per DNA ratio.

CDR3 size spectratyping (immunoscope)

The CDR3 size spectratyping was performed by TcLand (Nantes, France) as described elsewhere (Pannetier *et al.*, 1993).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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SUPPLEMENTARY MATERIAL

Figure S1A. GeneScan images from skin and blood of patients 1–10.

Figure S1B. GeneScan images from skin and blood of patients 11–20.

Figure S1C. GeneScan images from skin and blood of patients 21–31.

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