Clinical significance of T-cell clonality in mycosis fungoides and other cutaneous T-cell lymphomas

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Citation

Muche, J. M. (2010, May 20). *Clinical significance of T-cell clonality in mycosis fungoides and other cutaneous T-cell lymphomas*. Retrieved from https://hdl.handle.net/1887/15546

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Occurrence of circulating clonal T-cells in mycosis fungoides

Chapter 2 - Occurrence of circulating clonal T-cells in mycosis fungoides

2.1

Muche JM, Lukowsky A, Asadullah K, Gellrich S, Sterry W. Demonstration of frequent occurrence of clonal T cells in the peripheral blood of patients with primary cutaneous T-cell lymphoma. Blood. 1997; 90:1636-42

2.1 Demonstration of frequent occurrence of clonal T cells in the peripheral blood of patients with primary cutaneous T cell lymphoma

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Blood. 1997; 90:1636-42

Abstract.

Clonal T cells have been demonstrated in skin lesions of all stages of cutaneous T cell lymphomas (CTCLs). However, there are conflicting data regarding the CTCL stage in which dissemination of clonal cells into peripheral blood occurs. Although the multifocal occurrence of cutaneous CTCL lesions and T cell recirculation suggest an early appearance of neoplastic cells in the blood, circulating clonal T cells were only detected in advanced stages so far. We investigated their occurrence by a high sensitive PCR assay amplifying T cell receptor γ rearrangements and subsequent heteroduplex temperature gradient gel electrophoresis (HD-TGGE) of the amplification products. Circulating clonal T cells were found in 26/45 patients with mycosis fungoides (MF), in 6/7 with Sezary's syndrome (SS), in 10/13 pleomorphic CTCLs and 3/4 unclassified CTCLs. Corresponding skin specimens carried clonal T cells in 29/40 MF, 3/4 SS, 12/12 pleomorphic, and 2/2 unclassified CTCL patients. Except the blood specimen of a psoriatic patient, all samples of 60 controls (psoriasis vulgaris, atopic dermatitis and healthy volunteers) revealed polyclonal amplification products. In 30/32 CTCL patients carrying a clonal rearrangement in blood and skin, identity of both clones was indicated by HD-TGGE and confirmed by sequencing 6 of these cases. We found an unexpected high frequency of identical clonal T cells in peripheral blood and skin of CTCL patients, including early stages of MF. This supports the concept of an early systemic disease in CTCL and raises new questions concerning the pathogenesis.

Introduction.

Cutaneous T cell lymphomas (CTCLs) represent a heterogeneous group of non-Hodgkin lymphomas clinically originating in the skin and subsequently disseminating into lymph nodes, blood and other visceral organs.[1,2,3] According to the EORTC classification of primary cutaneous lymphomas, CTCLs are subgrouped into indolent (mycosis fungoides [MF], Sezary's syndrome [SS], pagetoid reticulosis, lymphomatoid papulosis, large cell CD30 positive CTCL), aggressive (large cell CD30 negative CTCL) and some provisional entities.[4]

Since it is well established that CTCLs are clonal expansions of T cells carrying identical copies of rearranged T cell receptor (TCR) genes, the demonstration of a predominant T cell clone in cutaneous infiltrates confirms the diagnosis additional to clinical, histopathological and immunophenotypic criteria. Southern blotting, displaying TCR mediated diversity of the restriction fragment length as well as more sensitive PCR assays, characterizing the V-(D)-J junction of TCR rearrangements, are applied to detect clonality.[5,6,7,8,9,10,11,12,13] Using a sensitive PCR assay, we recently demonstrated clonal disease in skin lesions of early MF.[14]

In addition to skin biopsy samples, extracutaneous specimens have often been analyzed by molecular biological techniques to investigate an extracutaneous spread of the CTCL. Regarding the peripheral blood, the majority of these studies demonstrated circulating clonal T cells only in SS and some cases of advanced stages of other CTCLs. In accordance to the clinical course of these entities, an association of blood involvement with poorer prognosis, lymph node involvement as well as an enlarged total body tumor burden was suggested. [7,9,15,16,17]

However, the frequent occurrence of multifocal or diffuse cutaneous CTCL lesions as well as the T cell nature of the malignant cell, emphasize a stage independent recirculation of the neoplastic cells via the peripheral blood to the skin. [1,18] For this reason, circulating clonal T cells should already be detectable in early stages of all CTCL types. Interestingly, early hematogeneous involvement in MF has been supposed by Bunn et al.[19]. However, the applied analytic techniques including E-rosette cytology, electron microscopy and cytogenetics possess a low diagnostic specificity and are considered to be of only complementary value in the diagnosis of CTCL.[20] By usage of a sensitive PCR-based method, the presence of circulating clonal T cells in early CTCL was demonstrated by Veelken et al.[21] in 2 patients with MF stage I. Additionally, Theodorou and colleagues[22] demonstrated clonality of blood samples in 47.2 % of 37 CTCL cases. Although a high frequency of blood involvement was discussed, this study lacked the differentiation between MF and SS which is well recognized to carry clonal T cells in the peripheral blood[23], and the different MF stages, respectively. In conclusion, data concerning occurrence and significance of blood clonality in CTCL are contradictory so far.

The aim of the present study was to investigate the occurrence of circulating clonal T cells in CTCL by applying a sensitive PCR/ HD-TGGE assay to blood and skin samples of a larger cohort of well classified CTCL. Special attention was paid to the analysis of early stages of MF.

Materials and Methods.

Patient samples. Blood specimens were obtained from 129 adult individuals: patients with CTCL (n=69), atopic dermatitis (AD, n=20), psoriasis vulgaris (PV, n=20) and healthy volunteers (HV,

n=20). Additionally, in 98 of the 129 patients a skin biopsy was analyzed (Table 1). No significant differences between the age of the control individuals (AD, PV, HV; range 43-76 years, median 61) and that of the CTCL patients (range 43-88 years, median 64) were found by the Mann-Whitney U test. The diagnosis was based on clinical criteria as well as histological and immunohistological assessment of formaldehyde fixed paraffin-embedded skin specimens.[24] CTCLs were classified according to the revised EORTC Classification.[4] The TNM classification was applied for further subgrouping of the MF cases.[24] Four cases of CTCL remained unclassified since they did not fulfill the criteria of any distinct CTCL entity. The cell lines JM (rearranged Vγ8 and Vγ11) and PEER (rearranged Vγ9) as well as peripheral blood of patient Ra suffering from γδ+ T cell acute lymphatic leukemia (T-ALL, Vγ10+) served as positive clonal controls.

Diagnosis	**Detection of clonality in**			
	blood specimens		**skin specimens**	
	n*	**%**	**n***	**%**
CTCL				
MF I A	6/ 13	46.2 %	9/ 12	75.0 %
MF I B	15/ 27	55.6 %	17/ 24	70.8 %
MF II- IV	5/ 5	100 %	3/ 4	75.0 %
Sezary's syndrome	6/ 7	85.7 %	3/ 4	75.0 %
Pleomorphic	10/ 13	76.9 %	12/ 12	100 %
Unclassified	3/ 4	75.0 %	2/ 2	100 %
total	45/ 69	65.2 %	46/ 58	79.3 %
Controls				
Psoriasis vulgaris	1/ 20	5.0 %	0/ 20	0 %
Atopic dermatitis	0/ 20	0 %	0/ 20	0 %
Healthy volunteers	0/ 20	0 %	n.t.	n.t.
total	1/ 60	1.7 %	0/ 40	0 %

Table 1. Investigated individuals and frequency of detection of clonal TCRγ rearrangements. *Number of specimens containing a dominant T cell clone vs. investigated samples. MF, mycosis fungoides; n.t., not tested.

Sample preparation. Peripheral blood mononuclear cells (PBMC) were prepared from 10 mL of heparinized blood by density gradient centrifugation through Ficoll-HyPaque (Pharmacia, Freiburg, Germany). Genomic DNA was prepared from about $1x10^6$ PBMC or JM/ PEER cells, respectively by a standard procedure using Proteinase K digestion.[25] For preparation of genomic DNA from the paraffin embedded skin specimens, the paraffin of 10 sections per sample (10 µm each) was dissolved with xylene. After centrifugation, the pellet was washed with ethanol and also digested by proteinase K.

TCRγ PCR. TCR-γ rearrangements were amplified using primers annealing at the V and J segments, respectively (Table 2). PCR 1 (primers VG1, VG2, VG9 and JG12-a) was applied to all specimens, whereas PCR 2 (primers VG1, VG2, VG9 and JGP12-a) was carried out in all control samples and those CTCL specimens appearing polyclonal in PCR 1. A primer for the JP segment was not included because JP is scarcely involved in TCRγ rearrangements.[26,27] In addition, a different J segment should be rearranged at the second allele. Reaction mixture included 0.5- 1 µg (5 µL) of genomic DNA, 1.75 U of Taq Polymerase and 7.5 µL 10x PCR buffer (Perkin Elmer, Branchburg, New Jersey,

USA), 0.1 mmol/L of each deoxynucleotide triphosphate (dNTP; Pharmacia, Freiburg, Germany), and 0.6 µmol/L of each primer in a final volume of 75 µL. Amplification was carried out on a thermal cycler (Varius-V; Vers, Hannover, Germany) by a 4 min. denaturation step at 95 °C, followed by 40 cycles including 1 min. denaturation at 94 °C, 1 min. annealing at 58 °C and 1 min. extension at 72 °C. Finally an extension step of 5 min. at 72 °C was added. Six µL of the PCR products were screened for successful amplification on a 2 % agarose gel stained by ethidium bromide.

Name	Primed segments	Position	Sequence (5' to 3')
VG1[a]	Vγ1, 2, 3, 4, 5, 5p, 6p, 7p, 8	104-121	CTCCATCCACTGGTACCT
VG9	Vγ9	121-138	ATTGGTATCGAGAGAGAC
VG2	Vγ10, 11, B, (A)	111-129/ 117-135	CACTGGTACKKGCAGAAAC
JG12-a[a]	Jγ1, 2	27-44	CAACAAGTGTTGTTCCAC
JG12-i	Jγ1, 2	20-37	TGTTGTTCCACTGCCAAA
JGP12-a	JγP1, P2	31-48	CTATGAGCYTAGTCCCTT
JGP12-i	JγP1, P2	16-35	CCTTYWGCAAAYRTCTTGA
VGseq	Vγ1, 2, 3, 4, 5, 5p, 6p, 7p, 8	136-153	AGRCCCCACAGCRTCTTC

Table 2. PCR and sequencing primers for TCRγ V and J genes. [a] adapted from Volkenandt et al.[10]

Determination of the clonality. The T cell clonality was established by detection of a dominant TCRγ rearrangement in a heteroduplex loaded temperature gradient gel electrophoresis (HD-TGGE). Eight µL of the PCR products were prepared to form heteroduplices (5 min denaturation at 95 °C, gradual cooling to 50 °C)[28] and separated on the Diagen TGGE-System (Diagen, Hilden, Germany). Electrophoretic run and subsequent silverstaining were performed according to standard protocols.[29] Evaluation of the gradient gels was done blindly by 2 independent investigators.

Due to the denaturation-renaturation step, polyclonal (i.e. not identical) amplification products form heteroduplices which contain mismatches in the N region. These mismatches decrease the thermal stability of the N region and alter the fragment migration. As a result, a broad smear on the gel is formed in this case. In contrast, clonal (i.e. identical) PCR products are expected to produce more stable homoduplices which migrate as sharp bands into the high temperature range of the gradient gel[12] (Fig. 1).

Cloning and sequencing of the TCRγ rearrangements. Thirty-two samples of CTCL patients and the clonal controls (JM, PEER, Ra) were sequenced directly and/ or after cloning of the PCR products. For direct sequencing, amplification products were separated by HD-TGGE. The distinct band was cut out and dissolved in 40 µL 1x PCR buffer (Perkin Elmer, Branchburg, New Jersey, USA) overnight. Five µL of the solution were reamplified at the same conditions described above. Primer JG12-i or JGP12-i was applied instead of JG12-a or JGP12-a, respectively (Table 2). The PCR product was purified by the QIAquick PCR purification kit (Qiagen, Hilden, Germany) and sequenced on an automated DNA sequencer (Model 373A, Perkin Elmer Applied Biosystems, Weiterstadt, Germany) by the Taq cycle sequencing method using primers VGseq, JG12-i, or JGP12-i (Table 2). Sequences were aligned to the published germline sequences of the TCRγ V and J segments.[30,31,32,33,34,35,36] Cloning of the PCR products was performed by applying the TA Cloning Kit (Invitrogen, Fleek, The Netherlands). Plasmids were sequenced using the universal forward

sequencing primer for M 13 by the method mentioned above. For each sample, 12 randomly chosen clones were analyzed. Identical TCRγ sequences repetitively found in the clones of one tissue sample were considered as the predominant T cell clone.

Results.

Validity of the diagnostic system. Ninety-nine of 100 PCR products from PBMC and skin specimens of the control groups (HV, PV and AD) formed a broad smear in the gradient gel (Table 1; Fig. 1, lanes 1 to 5, range a). In contrast, amplification products from the cell lines and from PBMC of a T-ALL patient (Ra) revealed a clear cut band below the observed smear (Fig. 1, lanes 6-9, range b). Accordingly, clonality of a PCR product was considered if a clear cut band appeared below the middle range of the temperature gradient gel (Fig. 1, lanes 10-15, range b). Except the cell lines, these cases revealed a smear of varying intensity above the sharp band representing the polyclonal background. In 10 samples (6 blood and 4 skin specimens), 2 sharp bands were observed in the gradient gel (Fig. 1, lanes 16 and 17, range b) indicating rearrangements of both TCRγ alleles in the T cell clone.[12] These cases were also classified as clonal.

To confirm the specificity of our PCR primers as well as the reliability of the HD-TGGE analysis, 26 clonal and 6 polyclonal PCR products were sequenced. All clonal amplification products revealed a TCRγ sequence (Table 3). The clonality of the determined sequence was proved by repeated analysis of independent PCR products obtained from a particular specimen (data not shown). In 17 clonal samples analyzed by both sequencing techniques, concordance was observed between direct sequencing and sequencing of multiple clones. The 6 polyclonal specimens (skin samples of patient Bo, Ti, Cz, Me; blood samples of patient Ja, La) were sequenced after cloning. Evaluation of at least 12 clones per specimen revealed different TCRγ sequences (data not shown).

The sensitivity of our PCR/ HD-TGGE system was determined by dilution of clonal T cells (JM cell line) in polyclonal PBMC of a healthy volunteer. After DNA preparation and amplification with primer VG1 and JG12-a, a distinct electrophoretic band was observed down to a dilution of 10^3 clonal JM cells in 10^6 PBMC, corresponding to a detection limit of 0.1 % clonal in polyclonal cells (Fig. 2, lane 5).

In conclusion, our diagnostic system revealed sufficient specificity and sensitivity.

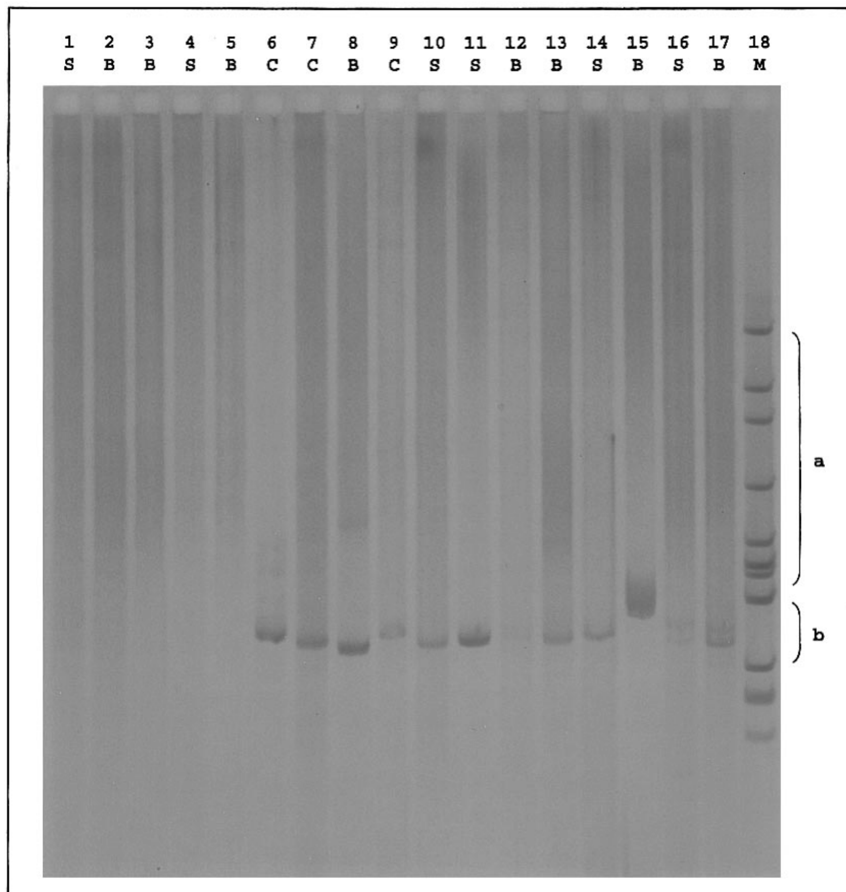

Figure 1. Temperature gradient gel of TCRγ PCR products. Lane 1-5: polyclonal PCR products of PCR 1 using primers VG1, VG2, VG9 and JG12-a (1-3) and PCR 2 using primers VG1, VG2, VG9 and JGP12-a (4, 5) appearing as broad smears in the middle range of the gel (a); lanes 6-9: clonal controls (cell line JM [Vγ8], cell line PEER [Vγ9], T-ALL patient Ra [Vγ10], cell line JM [Vγ11]) appearing as sharp bands below the middle range of the gel (b); lanes 10-17: clonal PCR products of PCR 1 (11-13) and PCR 2 (14, 15); lanes 16, 17: bialellic PCR products of PCR 1; lane 18: Hinc II digest of phi X174. S, skin sample; B, blood sample; C, cell line; M, marker.

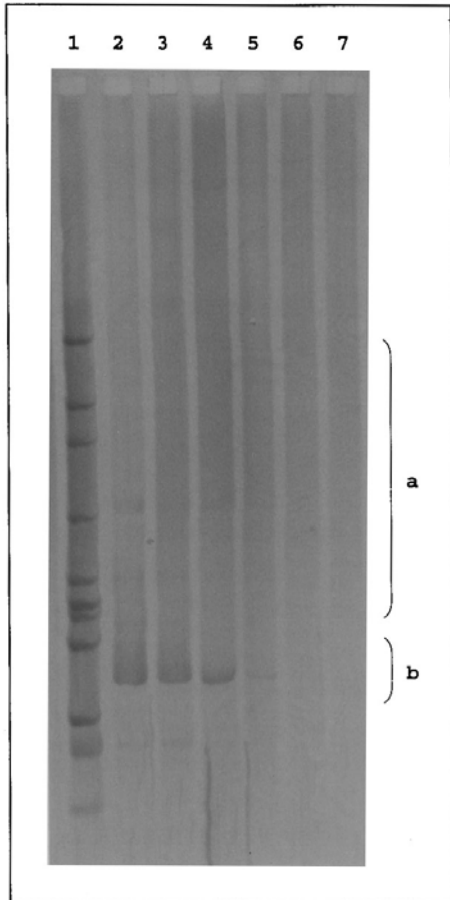

Figure 2. Temperature gradient gel of a dilution experiment. Lane 1: marker (Hinc II digest of psi 174); lane 2: 100 % JM cells; lanes 3-6: 10 %, 1 %, 0.1 %, 0.01 % JM cells in PBMC of a healthy volunteer; lane 7: 100 % PBMC. Clonality is demonstrated down to 10^3 JM cells in 10^6 polyclonal PBMC (0.1 %). a, range of polyclonal smears; b, range of clonal bands.

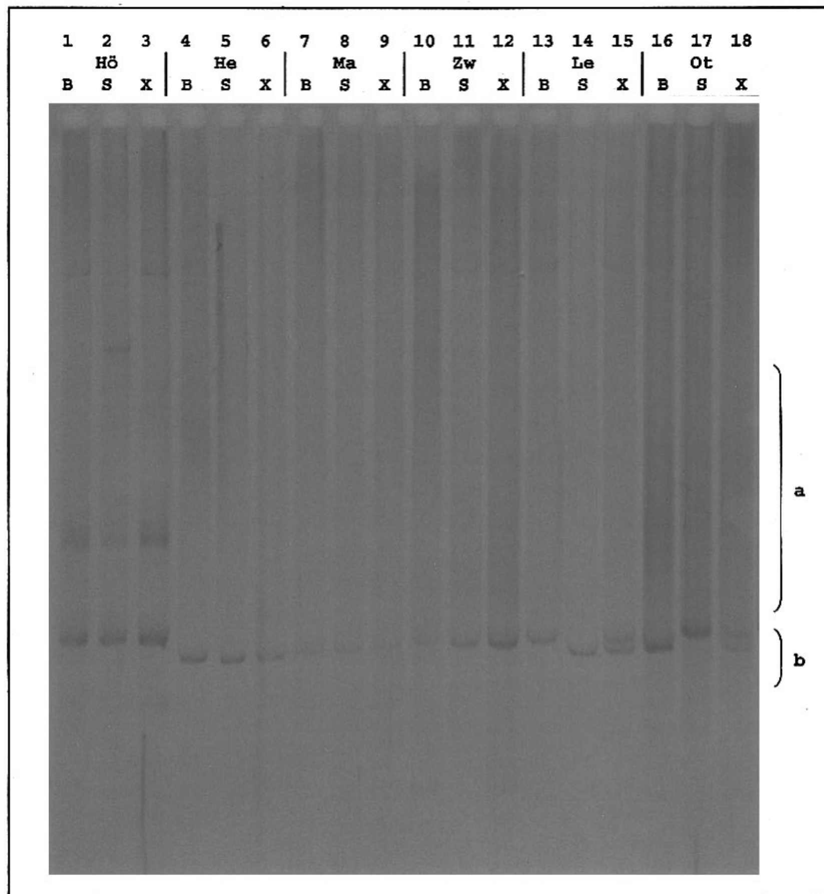

Figure 3. Temperature gradient gel of mixed corresponding clonal TCRγ PCR products.

S, skin sample; B, blood sample; X, mixture of blood and skin sample (1:1). Patients Hö, He, Ma, Zw show identical patterns in all 3 lanes; patients Le and Ot reveal different patterns, whereby lane X appears as summation of B and S. a, range of polyclonal smears; b, range of clonal bands.

Analysis of the blood specimens. A T cell clone was discovered in 45 out of 69 blood specimens obtained from CTCL patients (Table 1). Among these samples the lowest frequency of detected blood clonality was found in MF (57.8 %), whereas pleomorphic CTCL revealed clonal PCR products in 76.9 %, and SS in 85.7 % of the cases. Three of the 4 unclassified CTCLs yielded also clonal PCR products. Regarding the occurrence of circulating clonal T cells during progression of the disease, in MF stage I A 46.2 % of the cases were found to be clonal, whereas MF stage I B showed clonality in 55.6 % of the cases. All blood samples derived from MF stages II-IV revealed clonal PCR products. Blood samples of stage I were shown to carry clonal T cells significantly less frequent than the blood specimens of the more advanced stages II-IV characterized by skin tumors or erythroderma and/ or involved lymph nodes ($p<0.05$, chi square assay).

Among the 60 control specimens, the sample of a single psoriatic patient revealed a clonal PCR product (Table 1). Circulating clonal T cells were significantly more frequently detected in the CTCL

patients than in the controls ($p<0.001$, chi square assay).

Diagnosis	Pat.	Site	Sequ.		V-Segment	N-Region [a]	J-Segment	
MF I A	Ma	Sk	D + C	V8	ATTACTGTGCCACCTGGGAT	GAGC	AATTATTATAAGAAACTCTTTGG	J1/2
MF I A	Ma	Bl	D + C	V8	ATTACTGTGCCACCTGGGAT	GAGC	AATTATTATAAGAAACTCTTTGG	J1/2
MF I B	Ha	Sk	D + C	V2	ATTACTGTGCCACCTGGGACG	TACCTCGCCGGTAGG	AAGAAACTCTTTGG	J1/2
MF I B	Ha	Bl	D + C	V2	ATTACTGTGCCACCTGGGACG	TACCTCGCCGGTAGG	AAGAAACTCTTTGG	J1/2
MF II A	Hö	Sk	D + C	V2	ATTACTGTGCCACCTGGGACGGG	CCTTAGC	AAGAAACTCTTTGG	J1/2
MF II A	Hö	Bl	D + C	V2	ATTACTGTGCCACCTGGGACGGG	CCTTAGC	AAGAAACTCTTTGG	J1/2
MF II B	He	Sk	D + C	V2	ATTACTGTGCCACCTGGGACGGG	CTTGAGAC	TATAAGAAACTCTTTGG	J1/2
MF II B	He	Bl	C	V2	ATTACTGTGCCACCTGGGACGGG	CTTGAGAC	TATAAGAAACTCTTTGG	J1/2
LyPap	Zo	Sk	D	V2	ATTACTGTGCCACCTGGGACGG	–	ATTATTATAAGAAACTCTTTGG	J1/2
LyPap	Zo	Bl	D	V2	ATTACTGTGCCACCTGGGACGG	–	ATTATTATAAGAAACTCTTTGG	J1/2
pleoCTCL	Zw	Sk	D + C	V7p	ATTACTGTGCCACCTGGGA	ATTGCCCTTGGTGGTGGTTGGA	TTCAAGATATTTG	JP1
pleoCTCL	Zw	Bl	D + C	V7p	ATTACTGTGCCACCTGGGA	ATTGCCCTTGGTGGTGGTTGGA	TTCAAGATATTTG	JP1
MF I A	Ot	Sk	D + C	V2	ATTACTGTGCCACCTGGGACGGG	CTTGAGAC	TATAAGAAACTCTTTGG	J1/2
MF I A	Ot	Bl-1	D + C	V7p	ATTACTGTGCCAC	GACCTTTT	TTATTATAAGAAACTCTTTGG	J1/2
pleoCTCL	Le	Sk	D + C	V8	ATTACTGTGCCACCTGGGATAG	AA	ATTATAAGAAACTCTTTGG	J1/2
pleoCTCL	Le	Bl	D + C	V8	ATTACTGTGCCACC	CATTCATCTCCAATCCAA	AATTATTATAAGAAACTCTTTGG	J1/2
MF I A	Bo	Bl	C	V2	ATTACTGTGCCACCTGGGACG	TGCCGGGTTG	GAAACTCTTTGG	J1/2
MF I B	Ja	Sk	D	V2	ATTACTGTGCCACCTGGGACGGG	CTTGATACT	AGAAACTCTTTGG	J1/2
MF I B	La	Sk	C	V2	ATTACTGTGCCACCTGGGACGGG	CG	TTATTATAAGAAACTCTTTGG	J1/2
MF I B	Cz	Bl	D + C	V4	ATTACTGTGCCACCTGGGATGGG	CAA	ATTATAAGAAACTCTTTGG	J1/2
MF I B	Me	Bl	D + C	V7p	ATTACTGTGCCACCTGGGACAGG	CCCC	ATTATAAGAAACTCTTTGG	J1/2
LyPap	Ti	Bl	D + C	V7p	ATTACTGTGCCACCT	CCCTCGATGTATTATGGTG	TATTATAAGAAACTCTTTGG	J1/2
Cell line	JM	Cells	D (VG1)[b]	V8	ATTACTGTGCCACCTGG	AAATT	TTATTATAAGAAACTCTTTGG	J2
Cell line	PEER	Cells	D (VG9)[b]	V9	TACTACTGTGCCTT	CCGGCCCG	AAGAAACTCTTTGG	J2
T-ALL	Ra	Bl	D (VG2)[b]	V10	ACTACTGTGCTGCGTGG	GAGGGGT	TTATTATAAGAAACTCTTTGG	J1/2
Cell line	JM	Cells	D (VG2)[b]	V11	GGTGGTGTACCACTGTGCCTG	TCAGATCCTCACAGGGCGGGTT	TAAGAAACTCTTTGG	J1

Table 3. Sequences of dominant TCRγ rearrangements. [a] N region was determined according to Breit et al.[46]. [b] V primer used for sequencing. D, direct sequencing; C, sequencing after cloning; Sk, skin; Bl, blood; MF, mycosis fungoides; SS, Sezary’s syndrome; pleoCTCL, pleomorphic CTCL; LyPap, lymphomatoid papulosis.

Analysis of the skin specimens. In order to analyze whether the occurrence of circulating clonal T cells is associated with skin clonality, 98 simultaneously obtained skin specimens were analyzed.

Clonal PCR products were detected in 79.3 % of the CTCL patients (Table 1). The highest percentage of clonal skin samples was found in pleomorphic CTCL (100 %), whereas SS showed clonality in 75 %, and MF in 72.5 % of the cases. All cutaneous specimens of unclassified CTCL were demonstrated to be clonal. No clonal T cells were detected in the skin samples of the 40 controls (Table 1).

Analysis of corresponding skin and blood samples. In the 58 CTCL patients where skin and blood samples were taken simultaneously, clonal T cells in both compartments were found in 32 cases, whereas 8 revealed polyclonal rearrangements in blood and skin. Clonality was restricted to the skin in 11 of 40 MF cases and in 3 of 12 pleomorphic CTCL. In one out of 4 SS patients and in 3 out of 40 MF patients detection of a T cell clone was restricted to the blood. However, this phenomenon was not associated with any distinct MF stage (Table 4).

Diagnosis	n	Clonality in				No clonality in
		skin + blood		skin only	blood only	skin + blood
MF I A	12	5	(1)[a]	4	0	3
MF I B	24	10		7	2	5
MF II-IV	4	3		0	1	0
Sezary's syndrome	4	3		0	1	0
Pleomorphic CTCL	12	9	(1)[a]	3	0	0
Unclassified CTCL	2	2		0	0	0
total	58	32	(2)[a]	14	4	8

Table 4. Clonality of the corresponding blood and skin specimens. [a] Numbers in brackets indicate cases with clonal, but not identical rearrangement in both compartments. MF, mycosis fungoides.

In order to determine the identity of the T cell clones detected in blood and skin of the CTCL patients, PCR fragments from skin and blood samples were mixed and these mixtures were separated by HD-TGGE (Fig. 3). Thirty of the mixtures revealed migration patterns identical to those of the corresponding skin and blood samples. In 2 cases (patient Le, pleomorphic CTCL, and patient Ot, MF I A) two sharp bands were observed in the mixture lane. Each of these corresponded either to the band of the skin or of the blood sample, indicating different T cell clones in blood and skin of patients Le and Ot (Fig. 3, Table 4). To confirm the HD-TGGE results, samples of patients Le and Ot as well as 6 randomly chosen patients with identical TCRγ rearrangements, as detected by HD-TGGE, were sequenced directly and after cloning. In patients Le and Ot, different sequences of the dominating blood and skin rearrangements were determined. For the other 6 patients (Ma, Ha, Hö, He, Zo, Zw) identity of the clonal TCRγ rearrangements was verified by sequencing.

In summary, identical T cell clones were demonstrated in 51.7 % of all CTCL patients, including 33.3 % of MF I A, 41.6 % of MF I B, 75 % of MF II-IV, SS as well as pleomorphic CTCL and all unclassified CTCL cases (Table 4).

Discussion.

To evaluate an extracutaneous spread of malignant T cells in CTCL, several groups applied molecularbiological techniques. The majority of these studies detected circulating clonal T cells only in SS and some cases of advanced MF or pleomorphic CTCL. Therefore, it was postulated that blood

involvement is restricted to advanced cutaneous lymphoma and is associated with poorer prognosis, lymph node involvement as well as an enlarged total body tumor burden.[7,9,15,16,17] However, the frequent occurrence of multifocal or diffuse cutaneous CTCL lesions as well as the recirculational behavior of T cells[1,18] supports the hypothesis of an early occurrence of malignant T cells in the peripheral blood. Interestingly, early hematogeneous involvement in MF has been supposed by a few groups.[19,20,21,22] However, Bunn et al.[19] used techniques of low diagnostic specificity as E-rosette cytology, electron microscopy and cytogenetics[20], whereas the specific PCR based studies of Veelken et al.[21] and Theodorou et al.[22] lacked a sufficient number of analyzed cases and, respectively, a differentiation between MF and SS as well as a stage dependent analysis. Using a sensitive TCRγ PCR/ HD-TGGE system to investigate a large cohort of well classified CTCL, we demonstrated circulating clonal T cells in the majority of patients with MF and other CTCL. Surprisingly, this includes high frequencies of detected blood clonality in MF stage I (21/40) and pleomorphic CTCL (10/13).

PCR assays with subsequent high-resolution electrophoresis are well established for sensitive and specific detection of clonal TCR rearrangements in skin samples of CTCL patients as well as blood specimens of patients suffering from T cell leukemia.[37,38,39] However, the differences between the rearrangements are minimal and a high separation capacity of the electrophoresis is required. [27,40,41] We applied the HD-TGGE technique[12] to separate our PCR products. Determining the lower detection limit of our test system, we were able to discover up to 10^3 clonal in 10^6 polyclonal T cells (0.1 %).

On the other hand, increasing sensitivity might enable to detect minor clones of reactive lymphocytes in skin lesions of nonspecific dermatitis and cutaneous lymphoid hyperplasia proposed as the "clonal dermatitis" concept.[42] Moreover, there are some reports describing the detection of clonal T cell populations in peripheral blood, most notably CD8+ αβ T cells in healthy elderly donors.[43] However, our sensitive technique detected no clonality in 40 skin specimens of AD/ PV patients and in blood samples, only 1 out of 60 controls was found to be clonal. Statistical analysis revealed a significant higher frequency of clonality detection in the CTCL patients than in the controls, although there was no significant difference regarding the proband's age.

Moreover, we confirmed the specificity of our PCR/ HD-TGGE results by sequencing 26 clonal and 6 polyclonal amplification products. Applying two different strategies (direct sequencing and cloning with subsequent sequencing), a dominant TCRγ rearrangement could be demonstrated in all clonal cases analyzed, whereas polyclonal samples revealed different sequences after cloning.

Using HD-TGGE and sequencing, in 30 out of 32 CTCL patients carrying a clonal rearrangement in skin and blood compartment, identity of the dominating T cell clones was demonstrated. This includes 38.9 % of all MF stage I cases analyzed and 75 % of the investigated patients suffering from MF stage II-IV, SS, as well as pleomorphic CTCL. Therefore our findings sufficiently show that CTCL is a systemic and monoclonal disease right from the beginning, even if extracutaneous spread is not yet clinically apparent. With respect to the published data[7,16,17,21,22], this concept is supported by the association between the detection limit of the applied diagnostic method and the frequency of discovered clonality. It can be speculated that increasing sensitivity, i.e. clonospecific probes or primers, will enable to demonstrate circulating CTCL cells in almost all CTCL cases. Therefore, we

believe that the differences still observed in the frequency of blood clonality detection in the distinct CTCL stages are not of qualitative but of quantitative nature.

The detection limit of clones in our assay is about 0.1 %. Taking an average of 10^{10} T cells in the peripheral blood, we would calculate that there are approximately 10^{7} circulating CTCL cells in the 30 CTCL patients. This high quantity could suggest a systemic origin of CTCL. Further support in this direction comes from the findings in 4 CTCL cases (1 SS, 3 MF stage I/II) with T cell clones detectable in the peripheral blood but not in skin biopsies. Further analysis in the course of the disease will show whether these clones are the malignant cells responsible for the manifestation of the cutaneous lymphoma. It is also conceivable that these findings as well as the 2 cases with split clonality in skin and blood and the PV patient discussed in the results section are examples of T cell clonality occasionally detected in elderly persons [43], or indications of other malignancies such as initial T cell leukemia.

With respect to a recent data analysis, demonstrating a favorable long-term outcome of MF patients with clinical stage 1 A[44], blood clonality in the early stages as we demonstrated does not seem to be associated with a poorer prognosis. The explanation could be our observation of a high frequency of activated peripheral blood CD8+ T cells, suspected to be cytotoxic T cells, in the majority of early MF patients, indicating a considerable anti-tumor response.[45]

Despite the favorable course of MF I A, our results indicating the occurrence of circulating clonal T cells in early MF might support the concept of an early systemic treatment, i.e. interferon.

In summary, we demonstrated a high frequency of the occurrence of identical clonal T cells in peripheral blood and skin of CTCL patients, including early stages of MF. Our findings confirm the evidence for an early systemic disease in CTCL and, with regard to origin as well as dissemination of the cutaneous lymphoma cell, raise new questions concerning the pathogenesis of the disease. Longitudinal studies should gain further insight into this. Quantification of the circulating clonal cells might be useful as prognostic parameter for long time surveillance and as indicator of a therapy response.

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