Cutaneous T-Cell Infiltrates: Analysis of T-Cell Receptor γ Gene Rearrangement by Polymerase Chain Reaction and Denaturing Gradient Gel Electrophoresis

By Ioannis Theodorou, Marie-Hélène Delfau-Larue, Claude Bigorgne, Chantal Lahet, Gilles Cochet, Martine Bagot, Janine Wechsler, and Jean-Pierre Farcet

In cutaneous T-cell infiltrates, the demonstration of a clonal T-cell receptor (TCR) gene rearrangement has been considered helpful to distinguish Cutaneous T-cell lymphomas from reactive lymphoproliferation. Hence, a polymerase chain reaction (PCR) method using GC-clamp primers and denaturing gradient gel electrophoresis has been developed in our laboratory to analyze the TCRγ locus configuration. Two hundred eleven cutaneous samples from 155 patients were analyzed. A detectable clonal TCRγ rearrangement was significantly associated with cutaneous T-cell lymphomas as defined by morphologic and immunologic criteria. A clonal TCRγ rearrangement was also detected frequently in lymphomatoid papulosis, never in reactive lymphocytic infiltrates and B-cell lymphomas, and rarely in parapsoriasis en plaque and cutaneous lymphoid hyperplasia. Forty five patients had both a cutaneous and a peripheral blood sample. Fifteen had a detectable clonal rearrangement in the two samples and 22 were negative. Six patients had a positive skin sample and a negative blood sample, whereas two patients had a positive blood sample and a negative skin sample. Four lymph node samples were analyzed and the PCR results were the same as in the skin. Finally, 21 patients had sequential samples of recurrent skin lesions. The PCR results were concordant in all and, when detectable, the clonal TCRγ rearrangement remained unchanged in a given patient. Because of its simplicity and accuracy, the newly designed PCR procedure improves the monitoring of diagnosis, staging, and follow-up in cutaneous T-cell infiltrates.

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A NUMBER of cutaneous T-lymphoid infiltrates have the biologic potential to evolve into forms of disease, i.e., cutaneous T-cell lymphomas (CTCLs), that are more clinically aggressive. CTCL comprise various diseases whose diagnosis relies on clinical, morphologic, and phenotypic characteristics. However, none of these characteristics has a definitive specificity and CTCL diagnosis remains difficult especially in early or atypical cutaneous involvement. The demonstration of a predominant T-cell clone in skin biopsy has been proved helpful in the diagnosis of CTCLs as of other T-cell malignancies. The T-cell receptor (TCR) gene rearrangement provides a convenient genetic marker for the study of clonality in mature T-cell infiltrates. For example, the malignant cells in peripheral T-cell lymphoma express a TCRαβ in 60% to 70% of cases, a TCRγδ in less than 10% of cases, and neither TCR in up to 30% of cases. That a TCR is expressed or not, the γ locus is more frequently rearranged than the β locus. This finding is in agreement with the hierarchy of TCR gene rearrangement in T-cell ontogeny. Therefore, a single analysis of the TCRγ locus is sufficient to determine the clonality in peripheral T-cell lymphoma. In addition, the TCRγ locus rearrangement is suitable for PCR because the limited Vγ gene repertoire allows one to use a small number of oligonucleotide primers to analyze all Vγ gene combinations.

To analyze the TCRγ locus, a PCR procedure has been developed in our laboratory. Because there is no restricted usage of Vγ and Jγ genes in CTCL as in peripheral T-cell lymphomas, primers covering the entire γ locus were designed. These primers can be used in a single reaction. However, because of limited combinatorial and junctional diversity, the similar size of amplified Vγ fragments could result in false clonal bands when analyzed by a standard gel electrophoresis. The use of GC-clamp primers and denaturing gradient gel electrophoresis (DGGE) improves the PCR accuracy compared with methods previously reported for the γ gene. In DGGE, the PCR products are separated by melting difference in the polymorphic N sequence at the VJ junction rather than by size difference. Addition of a GC-clamp ensures that the N sequence is in the first melting domain of the PCR products, which results in a genetic imprint specific for every TCRγ allele.

The study of a series of 155 cases with cutaneous T-cell infiltrates is reported. Analysis of the γ locus configuration by the PCR procedure shows that a detectable clonal TCRγ rearrangement was significantly associated with CTCL. Lymphomatoid papulosis (LP) behaved as CTCL with regard to the high frequency of a detectable T-cell clone. A clonal TCRγ rearrangement could also be shown in a minority of cutaneous lymphoid hyperplasia (CLH) and parapsoriasis en plaque (PPP). In addition to its simplicity for the diagnosis of clonality, the method proves to be useful to monitor cutaneous T-cell disorder staging and follow-up, because the genetic imprint in each case remained unchanged in different tissues and at different time points.

MATERIALS AND METHODS

Clinical samples. 211 cutaneous samples were obtained by 4-mm-diameter punch biopsy samples from 155 patients with various skin disorders (Table 1). The studied cases were referred to the Department of Pathology in Henri Mondor’s hospital for first diagnosis or expertise. The diagnosis was assessed on fixed paraffin-embedded skin specimens and processed for hematoxylin-eosin-safran stained sections. Immunohistochemical analysis was performed to characterize the infiltrating cell type.

From Service d’Immunologie Biologique and INSERM U91, Hôpital Henri Mondor; and Services de Dermatologie and Départements de Pathologie, Hôpital Henri Mondor, Créteil, France.

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Address reprint requests to Pr. Jean-Pierre Farcet, MD, Service d’Immunologie Biologique, Hôpital Henri Mondor, 94010 Creteil, France.

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phenotyping was performed on frozen sections using an indirect immunoperoxidase technique. Monoclonal antibodies for cellular markers were as follows: CD2, CD3, CD5, CD7, CD8 (Becton Dickinson, Mountain View, CA), TCRβ chain/βF1, TCRγ TCRδ (T-cell Cambridge, MA), CD19/8, immunoglobulin light chain (Coulter, Hialeah, FL), CD68, CD30 (DAKO, Glostrup, Denmark).

CTCL were subclassified according to the updated Kiel classification in cutaneous pleomorphic small and medium-sized T-cell lymphomas (CTCL) and cerebriform lymphoma (mycosis fungoides (MF)/Sezary syndrome (SS)). All cases of MF were of patch or plaque stage. There were three cases of lymphoma (CPTCL) and cerebriform lymphoma (mycosis fungoides type A according to Willemze). PPP was diagnosed because of the skin biopsy in 45 patients, three of whom had an SS. The nomenclature of the Vy and Jy genes follows: CD2, CD3, CD5, CD7, CD8 (Becton Dickinson, Mountain View, CA), TCRβ chain/βF1, TCRγ TCRδ (T-cell Cambridge, MA), CD19/8, immunoglobulin light chain (Coulter, Hialeah, FL), CD68, CD30 (DAKO, Glostrup, Denmark).

Peripheral blood lymphocytes (PBLs) were obtained simultaneously from the skin biopsy in 45 patients, three of whom had an SS. A lymph node sample was available in four patients with MF.

**PCR amplification.** The nomenclature of the Vy and Jy genes is in accordance with Lefranc et al. V and J primers are described in Table 2. They could be mixed in a single reaction (multiplex PCR) to distinguish the allelic configuration of a rearrangement. DNA was prepared by a standard proteinase K digestion and a phenol/chloroform extraction. The reaction mixture included 250 ng of genomic DNA, 40 pmols of each primer, 1.5 U of Taq polymerase, 1 U of Uracil DNA glycosylase (GIBCO BRL, Gaithersburg, MD), 5 µL of 10x reaction buffer, 2.5 mmol/L MgCl2, and 200 µmol/L each of deoxyadenosine triphosphate (dATP), deoxyguanosine triphosphate (dGTP), deoxycytidine triphosphate (dCTP), and 400 µmol/L deoxyuridine triphosphate (dUTP) in a final volume of 50 µL. Samples were overlaid with 50 µL of mineral oil and transferred on the thermal cycler (model 480; Perkin Elmer Cetus, Norwalk, CT). To avoid contamination by the amplification products, they were first held at 50°C for 10 minutes to allow UDG to destroy any dUTP carried over. The reaction buffer contains amplified product that could have been carried over from previous reactions. This incubation was followed by a 10 minute inactivation of UDG at 94°C before cycling. Each cycle included a denaturation step (94°C for 1 minute), an annealing step (56°C for 1 minute) and an elongation step (72°C for 2 minutes). After 40 cycles, residual Uracil DNA glycosylase was inactivated by the addition of 50 µL of chloroform. Thirty microliters of the amplified products were run on a 6.5% polyacrylamide gel containing a linearly increasing 10% to 60% denaturing gradient (100% denaturant = 7 mol/L urea and 40% vol/vol formamide) in TAE buffer (40 mmol/L TRIS base, 20 mmol/L sodium acetate, 1 mmol/L Na2EDTA) pH, 7.4. During the run, the gels were immersed in TAE buffer held at 60°C in a gel apparatus similar to that described by Myers et al. This device fits gels 22-cm in length and was purchased from CBS Scientific Company (Prolabo, Paris, France). Gels were run at 150 V for 5 hours stained with ethidium bromide and photographed under ultraviolet illumination.

**Statistical analysis.** Comparisons of categorical data have been made using the Chi test or Fisher’s exact test when appropriate.

**RESULTS**

The TCRγ locus configuration was analyzed in a series of 211 cutaneous samples from 155 patients with various T-cell infiltrates (Table 1). As illustrated in Fig 1, the DGGE

| Table 1. Cutaneous Lymphoid Infiltrates From 155 Patients |
|----------------|-------------------|
| Diagnosis      | No. of Patients   |
| CPTCL          | 39                |
| MF/SS          | 45                |
| LP             | 10                |
| CLH            | 25                |
| PPP            | 7                 |
| RLI            | 21                |
| CBCL           | 8                 |

| Table 2. V and Jy Gene Primers for PCR |
|----------------|-------------------|
| Gene           | Primer Sequence   | Position   |
| Vγ1 (GC clamp) | CCAGAGAAACCACAG    | 368-387    |
| Vγ2 (GC clamp) | ATCTGAGCTACGGAA    | 360-381    |
| Vγ3 (GC clamp) | ACAAAGTGGAGGAAGAA  | 366-387    |
| Vγ4 (GC clamp) | GCTAGGTTGGGAAGACTA | 336-385    |
| J1             | CGATACTTAATTGCAGAC | 217-197    |
| J2             | TTGTCCCGGAACAAATACCTT | 75-54   |
| J3             | AGCTTAGCTCTTCACGATA | 71-50     |
| J4             | AGGCTAGCCTTTTTGCAACG | 60-39    |

The Vγ1 primer recognizes a conserved sequence of the Vγ family. The Vγ family is comprised of 8 genes and the indicated position corresponds to the primer sequence in the Vγ2 gene. Vγ2, Vγ3, and Vγ4 recognize the unique members of the respective families. A common primer for the J1 and J2 segments was designed and the position indicated is from the germline sequence of J2. All Vγ primers included the 5'-GCAGGGCCGGCCGGCCGGGGCGC-3' stretch.

![Fig 1. Electrophoretic imprint of VγJγ-amplified products is case specific. Lane 1, CPTCL; lanes 2 and 3, CLH; lanes 4 and 5, LP; lanes 6 and 7, MF; lanes 8 through 11, CPTCL; lanes 12 and 13, RLI; lane 14, normal skin.](image-url)
of the PCR products resulted in four clearcut patterns. A smear encompassing the bottom half of the gel was characteristic of VJ fragments amplified from polyclonal T-cell infiltrates with absence of a predominant clone (Fig 1, lanes 3 and 12 through 14). When a predominant T-cell clone was present, one or, more often, two bright bands with the same intensity superimposed to the smear. These bands, namely the homoduplexes, resulted from the amplification of monomeric and biallelic Vγγ rearrangements, respectively (Fig 1, lanes 1, 2, 4 through 7, and 9 through 11). Hence, the DGGE patterns in the cutaneous T-cell infiltrates were similar to those observed in the lymph nodes of peripheral T-cell lymphomas. Also similar to the DGGE in rare cases of peripheral T-cell lymphomas, the DGGE in CTCL could result in a three- or four-band pattern, the upper band being a heteroduplex (Fig 1, lane 8).

Table 3 shows the frequency of a detectable clonal TCRγ rearrangement in 211 skin samples analyzed in a 2-year period. When several simultaneous skin samples from the same patient could be analyzed, one sample could be negative while one or more others contained a detectable TCRγ rearrangement. Then, the patient was considered as positive. Accordingly, the frequency of a detectable clonal rearrangement was slightly higher when referred to the patients than referred to the samples (Table 3). In the 155 patients, the prevalence of monoclonality varied significantly (P < .001) according to the diagnosis of the cutaneous T-cell infiltrates. Four diagnostic categories were considered as follows: CTCL, LP, controls including the RLI and CBCL, and an intermediate category including CLH and PPP. Monoclonality was associated with the majority of CTCL and LP (Table 3), whereas none of the controls showed a clonal TCRγ rearrangement. CLH and PPP comprised a detectable T-cell clone in a minority of cases (Table 3).

The sensitivity of the PCR procedure was determined (Fig 2). The DNA from the Jurkat T-cell line with a biallelic VγI J1 and VγIV J1 rearrangement was diluted in DNA from normal skin. Under ultraviolet illumination, the VγI J1 electrophoretic band was visible at 1% and 0.1% dilution when the multiplex and monoplex PCRs were used, respectively. The sensitivity was even better for the VγIV J1 rearrangement (Fig 2). These results were similar to those obtained when DNA from the Jurkat cell line was diluted in DNA from normal peripheral blood mononuclear cells or from a reactive lymphadenopathy.

Forty-five patients had a blood and skin biopsy performed. Although 22 patients were negative for both samples, 15 had a detectable TCRγ rearrangement in both blood and skin. As illustrated in Fig 3, the electrophoretic band pattern was the same in the two tissues for every positive patient. Eight patients had PCR results which differed. Interestingly, two were positive in the peripheral blood and negative in the skin, whereas the reverse was observed in six patients. The two patients with a positive blood sample and a negative skin sample had SS. Altogether, peripheral blood samples with a detectable TCRγ rearrangement (n = 17) were observed in 9 of 17 MF/SS cases, 6 of 13 CPTCL cases, one of three LP cases and one of the two PPP cases, which were tested. Five blood samples in each of the two groups, RLI and CLH, were negative.
Four MF patients had a lymph node biopsy. A TCRy rearrangement, identical to that in the skin (Fig 3), was shown in three of four lymph nodes. The fourth case was negative in both the skin and lymph node sample.

Finally, 21 patients had two or more sequential biopsies for recurrent skin lesions performed. This group included 12 CPTCL, 8 MF, and 1 LP patients. Among 12 CPTCL patients who relapsed, 11 had positive sequential samples with a genetic imprint stable and specific for every patient (Fig 3). One of these 12 patients remained negative despite four sequential samples of recurrent skin lesions that were analyzed. The results of sequential analyses were also concordant in 8 patients with MF; 5 patients being positive and 3 patients negative for all samples. Of the 21 patients tested in the 2-year interval of this study, only 1 patient with an LP had first a negative sample, and 3 months later, a positive sample.

**DISCUSSION**

The diagnosis of CTCL is often hampered by the difficulty to distinguish neoplastic from reactive T lymphocytes using conventional morphologic and immunophenotypic studies. Progress has been made by the possibility to analyze TCR gene rearrangements and the demonstration that neoplastic T cells are clonal in origin. In addition, analysis of TCR gene rearrangements improves CTCL staging accuracy and allows one to assess whether occult disease is present during clinical remission. Because of its simplicity and rapidity, PCR has been recognized to be more effective than Southern blot to analyze the TCR locus configuration. Most groups, including ours, analyze the TCRy locus whose germ line V gene repertoire is limited. The PCR procedure developed in our laboratory offers two advantages. The primers covering the entire y locus can be used in a single reaction and the DGGE combined to the GC clamp ensures that the VJ-amplified products are separated on melting difference in the VJ joining N region. The GC-clamp at the 5′ end of V primers has a higher fusion temperature equivalent, which places the polymorphic N sequence in the first melting domain of PCR products. Consequently, on DGGE, the migration of PCR products is selectively retarded by the fusion of double-strand DNA in the N region, which results in a genetic imprint highly specific of every TCRy-rearranged allele.

The PCR was used to analyze the y locus configuration in skin biopsy samples of 155 patients with cutaneous T-cell lymphoid infiltrates. Evidence for a clonal TCRy rearrangement was significantly associated with the neoplastic T-cell proliferation in CPTCL and MF. This is in agreement with previous reports of limited series of patients studied by either cyogenetic or Southern blot. Recently, two groups using PCR and TGGE or DGGE, but not the GC-clamp, reported similar findings. Although we analyzed all VJy genes possibly rearranged including Vy III, Vy IV, Jy1, Jy2, and Jy genes in addition to Vy I, Vy II, and Jy I/II genes studied by these two groups, a sizeable number of CPTCL and MF cases had a polyclonal electrophoretic pattern. In these cases, three explanations should be considered for the absence of a predominant TCRy rearrangement. Firstly, the number of clonal neoplastic T cells were below the threshold of the PCR sensitivity. In a few cases, this could be caused by a punch biopsy that did not involve enough neoplastic T cells, as suggested by the presence of a detectable clone in other skin and peripheral blood specimens from the same patient. Secondly, neoplastic T cells could be oligoclonal as recently reported in a cytogenetic study. In the latter, it was found that chromosomal abnormalities were oligoclonally distributed in 17% of MF. Oligoclonality and small number of malignant T cells could combine resulting in a cutaneous infiltration by several neoplastic clones of small size that cannot be distinguished from normal reactive T cells. A third explanation would be that the TCRy locus was in germline configuration or that a transrearrangement, namely Vy+(D/p) Jy, has described as in normal T-cells. That the number of MF cases with a detectable clonal TCRy rearrangement was higher (90%) in the series by Wood et al than in ours can be caused by patient selection and technique differences. Our group of MP patients include only patients with patches and plaques and no patients at a tumoral stage. With regard to the PCR procedure, we used 10 times less DNA for amplification, a single mix with all VJ primers and a single-step amplification instead of a nested PCR. Nevertheless, the sensitivity of the two PCR procedures appears to be in the same range. They could detect a dominant clone constituting as little as 1.0% to 0.1% of the total cells in the sample (see Fig 2).

A clonal TCRy rearrangement was evidenced in 7 of 10 patients with LP. The prevalence of monoclonality in LP was statistically similar as that in CTCL. This finding is in agreement with the results of Southern blot studies in LP and LP is known to be associated with CTCL in 10% to 20% of patients and the same TCR rearrangement was shown in the two types of cutaneous lesions 16 years apart in 1 case. PPP and CLH have a benign clinical and histologic presentation as LP. In contrast with LP, the PCR analysis disclosed infrequently a clonal TCRy rearrangement, ie, in 1 of 7 PPP and 3 of 25 CLH. To determine whether the patients with a detectable T-cell clone are those who evolve into CTCL after many years would require a long prospective follow-up. However, occasional cases have been reported in which the TCR rearrangement characteristic of the malignant cells in CTCL samples were traced back in early PPP and CLH T-cell infiltrates.

The specificity of TCR gene analysis has improved CTCL staging accuracy. Using Southern blot, neoplastic T cells were shown in peripheral blood and lymph nodes, whereas morphologic and immunophenotypic studies remained unconvincing. Using PCR, which is more sensitive and facilitates multiple analyses, the same TCRy rearrangement was evidenced in both peripheral blood and skin of 15 patients studied. Whether peripheral blood involvement evidenced by PCR is related to an advanced disease and of poor prognostic value or corresponds to an early systemic circulation of malignant T cells should await a prospective study. Interestingly, two additional patients had a positive PCR in peripheral blood, whereas it was negative in the skin. It should be emphasized that the three patients with SS had a positive PCR in peripheral blood and that two of them were simultaneously negative in the skin. These data show...
that peripheral blood analysis is also of diagnostic value, especially in erythrodermic patients. Four lymph node biopsy samples in MF patients were available. The lymph node analysis by PCR showed a predominant T-cell clone in three of four biopsies, which was the same number as in the skin. In agreement with previous studies using Southern blot, the PCR results strongly suggest that neoplastic T cells have involved a palpable lymphadenopathy even when the lymph node sample looks histologically benign. Therefore in the case with absence of a predominant T cell clone in both lymph node and skin samples, oligoclonal feature of neoplastic T cells should be considered.

Because of its simplicity and accuracy, the PCR facilitates follow-up study. Twenty-one patients had two or more skin biopsies during the 2-year interval of the present study. Twenty patients had CTCL. All of the CTCL patients had concordant PCR results, either positive or negative, along this interval. This raises the possibility that the clonal or oligoclonal feature of neoplastic T cells might be an early feature of the disease, though a much longer follow-up interval is required before drawing such a conclusion. Nevertheless, the specificity of the PCR procedure reinforces the concept that the clonal TCR gene rearrangement, when present, remained unchanged over the years. This result allows one to envisage the monitoring of minimal residual disease, if necessary, for the management of CTCL treatment. The sensitivity and specificity can be increased by hybridizing the PCR products with a labeled oligonucleotide probe specific for the TCRγ rearrangement evidenced at diagnosis. This strategy will detect 10^-4 or less malignant cells in peripheral blood samples.

In conclusion, the proposed PCR procedure improves in several ways the monitoring of patients with cutaneous T-cell infiltrates. In addition to morphologic and immunophenotypical features, a rapid and accurate argument of clonality can be obtained for the diagnosis of CTCL. The CTCL staging is simplified by the possibility of analyzing several biopsy samples from different skin lesions as well as blood and other tissue samples when necessary. Finally, the specificity of the PCR allows a reliable diagnosis of relapse in recurrent skin lesions, and eventually the follow-up of minimal residual disease in peripheral blood when skin lesions are in clinical remission.

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