Conformational Polymorphisms of cRNA of T-Cell–Receptor Genes as a Clone-Specific Molecular Marker for Cutaneous Lymphoma

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A novel molecular assay for the detection and characterization of monoclonal lymphoid populations in clinical specimens was developed. The assay is based on the principle that upon non-denaturing polyacrylamide gel electrophoresis RNA molecules separate into several metastable conformational forms. These conformational polymorphisms strictly depend on the nucleotide sequence of the individual molecule. Using DNA from formalin-fixed, paraffin-embedded tissue of patients with mycosis fungoides, highly variable junctional sequences of rearranged T-cell receptor gamma genes were amplified by polymerase chain reaction. Subsequently, the polymerase chain reactions products were transcribed into complementary RNA and analyzed by non-denaturing polyacrylamide gel electrophoresis. In clinical specimens with a monoclonal lymphoid population, a clone-specific pattern of bands was identified representing conformational polymorphisms of cRNA molecules of rearranged T-cell receptor gamma genes of the predominant lymphoid clone. Three biopsies from one patient taken from different sites of the body over 3 years yielded an identical pattern of bands. This methodology provides a novel and rapid tool for the molecular identification and characterization of clonal lymphoid populations in clinical specimens. It is likely to be of special value for studies on the clonal evolution of lymphoid disorders of the skin. Key words: lymphoma/polymerase chain reaction/clonality detection/T-cell–receptor genes. *J Invest Dermatol 101:514–516, 1993*

The detection of a monoclonal lymphoid subpopulation in a clinical specimen is of considerable diagnostic interest and suggestive of a malignant lymphoproliferative disorder. On the molecular level, monoclonal lymphoid populations are traditionally detected by Southern blot analyses of T-cell receptor (TCR) or immunoglobulin gene rearrangements [1–4]. However, in dermatology, Southern blot analyses are not feasible in many clinical situations, because relatively large amounts of well-preserved DNA are required, which are often difficult to obtain, e.g., from small skin biopsies or from formalin-fixed, paraffin-embedded material. In addition, Southern blot analyses are labor and time intensive.

Recently, alternative approaches for the detection of clonal lymphoid proliferations have been reported [5,6]. These assays are based on the *in vitro* amplification by polymerase chain reaction (PCR) of rearranged sequences of immunoglobulin or TCR genes. For example, the junctional regions of rearranged TCR-gamma genes differ in size and nucleotide sequence between individual lymphocytes. The nearly unlimited junctional variability is created by small deletions of n cleotides during the process of rearrangement of variable and joint segments and by insertion of random nucleotides between rearranging sequences [7–9]. When DNA from polyclonal lymphoid cells is amplified and analyzed by gel electrophoresis, only a smear is visible, representing junctional regions of rearranged TCR-genes of numerous different sizes. However, when DNA from a clinical specimen with a monoclonal infiltrate of lymphoid cells is analyzed, a distinct band is visible, representing the junctional region of rearranged TCR-gamma genes of the dominant cell population. Although these PCR-based assays, as described so far, can indicate the presence of a monoclonal subpopulation, interpretation of the data is not always possible unambiguously, as lymphoid populations are detected based on size discrimination of the PCR products alone. Furthermore, these assays do not lead to a molecular characterization of the specific monoclonal lymphoid population of the malignant lymphoma of a particular patient. Thus, unlike Southern blot analyses of lymphomas, where a clone-specific band pattern is identified by hybridization procedures, these PCR-based assays will not allow the comparison of lymphoid clones in biopsies from different sites of a patient or taken at different time points during the course of the disease. Studies on the clonal evolution of lymphomas cannot be performed and the emergence of a second malignant clone in a patient will easily remain undetected. A recently developed technique¶ [10] involves the PCR amplification of rearranged TCR-gamma genes followed by fractionation of the PCR products by denaturing gradient gel electrophoresis (DGGE). With this type of gel electro-

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Abbreviations: DGGE, denaturing gradient gel electrophoresis; MF, mycosis fungoides.

¶Wood GS, Liao S, Carol F, Crooks CF, Sklar J: Cutaneous lymphoid infiltrates: analysis by polymerase chain reaction and denaturing gradient gel electrophoresis (PCR/DGGE) (abstr). *J Invest Dermatol* 98:553, 1992.

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phoresis PCR products are separated based on their size and nucleotide sequence. Monoclonal lymphoid populations result in one or more discrete bands in the gel and have been detected reproducibly by this technique.

We herein describe a novel methodology that detects clonal lymphoid subpopulations in clinical specimens by analysis of PCR products based on their nucleotide sequence. This technique leads to the identification of a specific band pattern upon polyacrylamide gel electrophoresis (PAGE), which is characteristic for the clonal tumor cell population. This assay is based on the principle that the conformational polymorphisms of RNA molecules strictly depend on the nucleotide sequence of the individual molecule. Upon non-denaturing PAGE, RNA molecules, which can assume elaborate secondary and tertiary structures, separate into several metastable conformational forms. We and others recently found that even single base pair mutations change the distribution and the number of these forms, which are characteristic for the particular nucleotide sequence of the RNA molecule [11,12]. In the present study, highly variable junctional sequences of rearranged TCR-gamma genes are amplified by PCR using primers that bind to conserved regions of variable and joint segments of the gene. Subsequently, transcription of the PCR products into complementary RNA (cRNA) is easily accomplished with the use of a T7 polymerase promoter sequence added to the 5′ end of one of the PCR primers. In clinical samples with a monoclonal lymphoid subpopulation, the cRNA molecules yield a clone-specific pattern upon non-denaturing PAGE. This pattern represents conformational polymorphisms of cRNA molecules of rearranged TCR-gamma genes of the predominant lymphoid clone.

MATERIALS AND METHODS

Clinical Samples Eight formalin-fixed, paraffin-embedded skin biopsies from five patients with mycosis fungoides (MF) were studied. One patient had three biopsies taken from different sites of the body over 3 years. From another patient two biopsies had been taken over 2 years. In addition, a biopsy from a patient with a T-cell pseudolymphoma was obtained. Each biopsy had a size of approximately 1 × 1 cm. Seven adjacent 15-μM-thick sections were cut from each paraffin block and placed into a 1.65-ml microcentrifuge tube. DNA was extracted as described [13,14]. Briefly, 1 ml of xylene was added to dissolve the paraffin. The pellet was washed once with 100% ethanol and twice with 70% ethanol and resuspended in 100 μl of digestion buffer (0.2 M Tris-HCl, pH 8; 10 mM ethylenediaminetetraacetic acid, 1% sodiumdodecylsulfate) to which Proteinase K was added to a final concentration of 0.5 mg/ml. After 24 h incubation at 55°C, the DNA was phenol-chloroform extracted and ethanol precipitated. After washing twice with 70% ethanol, the pellet was resuspended in 100 μl of filtered and autoclaved water. Three μl of each sample were used as template for PCR amplification reactions. Preparation and analysis of samples were performed in a blinded fashion without knowing which biopsies were taken from which patients.

***In Vitro* DNA Amplification Reactions** Highly variable junctional regions of rearranged TCR-gamma genes were amplified by PCR using consensus oligonucleotide primers annealing to conserved regions within the variable group I genes (primer $Vari_{cons}$) and to conserved sequences of TCR-gamma joint 1 and 2 genes (primer $Joint_{cons}$) [7,15]. A T7 RNA polymerase promoter sequence was added to the 5′ end of primer $Vari_{cons}$. Primer sequences were primer $Vari_{cons}$, TAA TAC GAC TCA CTA TAG GGA GCC AGG GTT GTG TTG GAA TCA (T7 RNA polymerase promoter sequence underlined) [16]; primer $Joint_{cons}$, GAC AAC AAG TGT TGT TCC AC. PCR amplification reactions consisted of 2.5 U Taq DNA polymerase (Perkin Elmer Cetus), 25 pmol of each primer, 200 μM of each dNTP, 3 μl of the DNA solution of each sample, and a 1 × reaction buffer as supplied by the manufacturer in a volume of 50 μl overlaid with mineral oil. Amplification conditions in a Perkin Elmer Cetus "Thermal Cycler" were 45 cycles of 94° for 1 min, 55° for 30 seconds, 72° for 30 seconds.

Transcription of the PCR Products into cRNA After PCR, 15 μl of the reaction mixture were added to 2.5 μl of a 10× transcription buffer (400 mM Tris/HCl, pH 8.0, 120 mM $MgCl_2$, 100 mM dithiothreitol, 10 mM spermidine), 2.5 μl of a 10 mM solution of ribonucleotides (ATP, CTP, GTP, UTP), 0.25 μl of RNAsin (Promega, WI), 0.6 μl of T7 RNA polymerase (69 U/μl, Pharmacia, Piscataway, NJ), and DEPC treated water to a final volume of 25 μl. 0.5 μl of 35S adenosine triphosphate (1494 Ci/mmol) was added and the mixture was incubated for 1 h at 37°C. To inactivate the enzyme, 0.75 μl of 0.5 M ethylenediaminetetraacetic acid was added.

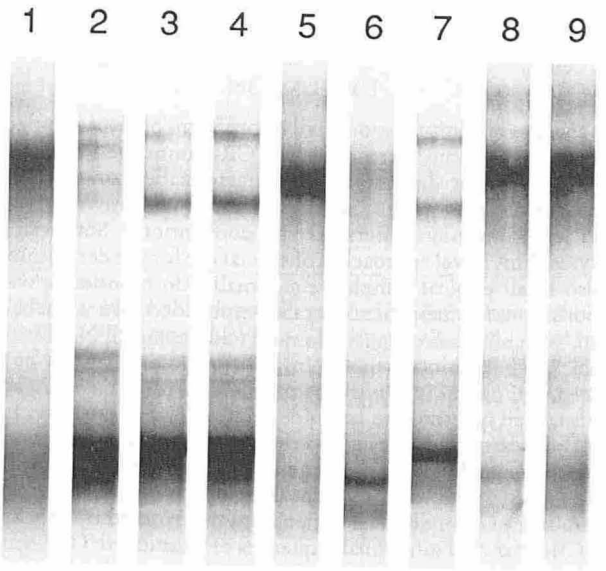

Figure 1. Analysis of conformational polymorphisms of cRNA molecules of junctional regions of rearranged TCR-gamma genes from eight formalin-fixed, paraffin-embedded skin biopsies of patients with mycosis fungoides (MF) (samples 2–9) and a patient with a T-cell pseudolymphoma (sample 1). Junctional regions of rearranged TCR-gamma genes were amplified by PCR, transcribed into cRNA, and analyzed by non-denaturing PAGE.

Gel Electrophoresis After the transcription reaction, 14 μl of the mixture was added to a new tube containing 3.5 μl of a loading buffer (50% glycerol, 0.25% bromphenolblue). After heating to 65°C for 7 min, the mixture was loaded onto an 8% polyacrylamide gel (acrylamide/bisacrylamide 19:1) containing 10% glycerol and electrophoresed at 16°C in 1 × TBE buffer for 3-4 hours at constant wattage (25W). After electrophoresis, the gel was dried and exposed to a Kodak XAR5 film overnight.

RESULTS

Analysis of cRNA molecules of junctional regions of rearranged TCR-gamma genes of all samples from patients with MF revealed several distinct bands upon PAGE (Fig 1, samples 2–9). These bands represent conformational polymorphisms of cRNA of the PCR-amplified junctional region of TCR-gamma genes of the predominant lymphoid clone. In addition, a background smear is visible, representing conformational polymorphisms of junctional regions of rearranged TCR-gamma genes of polyclonal T lymphocytes present in the lesion. When the pattern of bands visible on PAGE was compared between samples, samples 3, 4, and 7 were identical; samples 8 and 9 also revealed an identical pattern. Comparison of these results to the clinical data disclosed that samples 3, 4, and 7 were biopsies from the same patient taken within a time period of 3 years. Samples 8 and 9 were biopsies from another individual patient taken at a time interval of 2 years. Samples 2, 5, and 6, which showed different band patterns on PAGE, were biopsies from three different patients with MF. Sample 1, with no clearly identifiable band pattern, was a biopsy from a patient with a T-cell pseudolymphoma. Only a broad smear is visible, representing conformational polymorphisms of numerous different cRNA molecules. Similar smears with accentuations at nearly identical positions on the gel were observed, when other samples with a polyclonal lymphoid infiltrate, e.g., skin biopsies from patients

with lupus erythematosus or DNA from peripheral blood lymphocytes of healthy volunteers, were analyzed (data not shown).

DISCUSSION

The analysis of conformational polymorphisms of cRNA molecules of junctional regions of rearranged TCR-gamma genes is suitable for the molecular detection and characterization of monoclonal lymphoid populations in clinical specimens and provides a more rapid and convenient alternative to conventional Southern blot analyses. This novel approach is of special use for the dermatologist, as also small clinical samples, e.g., small skin biopsies as well as sections from formalin-fixed, paraffin-embedded tissues can be analyzed, which both frequently do not yield enough DNA for traditional Southern blot analyses. In addition, the assay can be performed without radioactivity, because a band pattern on PAGE can be visualized by ethidium bromide staining (data not shown).

In the present study we extend and further develop our concept of using rearranged TCR-gamma genes for the molecular analysis of lymphomas. Previously [15], a molecular marker sequence for the lymphoma of an individual patient was determined by subcloning of PCR-amplified junctional sequences of rearranged TCR-gamma genes into bacteria and subsequent sequence analysis of individual clones. In the present study, monoclonal lymphoid subpopulations in formalin-fixed, paraffin-embedded lymphoma specimens of individual patients are characterized solely based on a PCR amplification reaction, transcription into cRNA, and gel electrophoresis.

The cRNA conformational polymorphisms of highly variable rearranged genes represent a unique molecular "fingerprint" for individual lymphocytic clones and allow the comparison of biopsies from different sites of the body, as well as biopsies taken at different time points of the disease. The cRNA conformational polymorphisms result in considerably more bands upon non-denaturing PAGE (usually 5–10 bands) than analysis of PCR products by DGGE or analysis of genomic DNA by Southern blot techniques. This may be of advantage, as the molecular "fingerprint" provided by the cRNA based assay might be even more sensitive to nucleotide sequence differences in the amplified gene segment resulting in a change of the position of individual bands upon PAGE.

When analyzing a clinical sample based on cRNA conformational polymorphisms the possibility that the pattern of bands observed might represent polymorphisms of more than one lymphoid clone cannot be excluded entirely. In these cases, initial analyses of the PCR products (prior to transcription into cRNA) by PAGE are likely to indicate a possible biallelic rearrangement or a biclonal lymphoid population by resulting in several distinct bands upon PAGE. However, even in cases where the pattern of bands of cRNA conformational polymorphisms represents more than one rearrangement, the emerge of an additional clone is likely to become obvious when the pattern of bands between samples (e.g., of an individual patient) are compared.

Frequently, in patients with a T-cell lymphoma of the skin, enlarged lymph nodes or cutaneous lesions, which develop during the long course of the disease, are difficult to classify. Using this assay, a proliferation of the respective malignant lymphoid clone of the lymphoma of the patient can be identified. Furthermore, this assay will allow studies of the clonal evolution of lymphoproliferative disorders. For example, considerable controversy surrounds questions about the clonal evolution of lymphomas in patients with pre-existing lymphomatoid papulosis. It is still unclear whether clonal lymphoid cells of a malignant lymphoma in patients with long-standing lymphomatoid papulosis derive from T-cell populations in pre-existing lesions of lymphomatoid papulosis. Recently, using time-consuming DNA sequence analyses, a clonal relationship between lymphomatoid papulosis and Ki-1–positive large-cell anaplastic lymphoma has been shown [17], but larger studies using a more rapid technology, such as the present one, are needed. In addition, by analyzing junctional regions of rearranged immunoglobulin genes, the novel approach presented in this study may also be applied to the molecular analysis of B-cell lymphoid proliferations of the skin.

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