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Technical Advance

Analysis of T Cell Receptor-γ Gene Rearrangements by Denaturing Gradient Gel Electrophoresis of GC-Clamped Polymerase Chain Reaction Products

Correlation with Tumor-Specific Sequences

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We describe a modified denaturing gradient gel electrophoresis (DGGE) procedure with a 40-nucleotide GC clamp in the polymerase chain reaction to improve resolution in amplifying T cell receptor-γ (TCR-γ) rearrangements. DNA from 46 cases of lymphoblastic leukemia/lymphoma, 5 T cell lines, 2 B cell lines, 7 normal lymphocytes, and 3 cases of Hodgkin's disease was amplified by polymerase chain reaction. In addition, 20 cases of paraffin-embedded T cell lymphomas and 5 cases of reactive hyperplasia were also studied. Clonal TCR-γ rearrangements were identified on DGGE by the presence of a predominant band. Results obtained from 5 T cell lines and 12 lymphoblastic leukemia/lymphomas containing known TCR-γ gene rearrangements revealed 100% concordance in detecting clonal rearrangements between DGGE and traditional Southern blot analysis. Of the remaining 34 lymphoblastic leukemia/lymphoma cases studied by DGGE alone, 30 were positive. DGGE analysis of 10 lymphoblastic leukemia/lymphoma cases with known group I Vγ to Jγ1 or Jγ2 rearrangement sequences confirmed that the electrophoretic migration was dependent on the tumor-specific rearranged TCR-γ sequence. In addition, 17 of 20 cases of paraffin-embedded T cell lymphomas were positive by DGGE, 6 of which had the clonal population also identified in fresh tissue DNA. DGGE analysis of GC-clamped polymerase chain reaction products can provide a way to more accurately detect TCR-γ clonality of lymphoid tumors and can be applied to archival tissues. (Am J Pathol 1995, 146:46–55)

The detection of T cell receptor (TCR) gene rearrangements has been performed by Southern blot analysis for many years. In many laboratories, only TCR-β gene rearrangements have been reported in the diagnostic evaluation of clinical cases.[1] Probes for TCR-δ and TCR-γ have been infrequently used, the latter due to the reported difficulty of discriminating clonal gene rearrangements from polyclonal T cells.[2] This has been attributed to the limited diversity of Vγ genes available for rearrangement compared with other TCR.[3]

The diagnosis of T cell lymphomas is more difficult in paraffin-embedded tissues than B cell lymphomas

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as there is no analogous immunohistochemical study to demonstrate T cell clonality such as anti-κ and anti-λ antibodies for B cell neoplasms. Specific anti-TCR antibodies are available to only a fraction of all Vα, Vβ, and Vγ gene products. Diagnosis is especially difficult in those T cell lymphomas composed of cells with small, round to slightly irregular, nuclei and also those lesions in which the malignant infiltrate is present in a polymorphous background composed of plasma cells, histiocytes, and eosinophils. The latter morphology can be easily confused with a reactive process or, less commonly, Hodgkin's disease. If frozen tissue is available, immunoperoxidase studies can be performed to look for dropped T cell antigens such as CD2, CD3, CD7, or CD5 or an aberrant immunophenotype of CD4 and CD8. When frozen tissue is not available for Southern blot analyses, frequently a new biopsy is recommended.

Recently there have been a number of reports describing the use of the polymerase chain reaction (PCR) to amplify and detect clonal TCR gene rearrangements including TCR-β,[4] TCR-δ,[5–7] and, most frequently, TCR-γ.[8–15] The limited number of V genes for TCR-γ makes it an easier system to select and optimize primers than the TCR-β gene, which has over 20 Vβ regions.[4,15] Amplified TCR PCR products have been analyzed by routine agarose gel electrophoresis (AGGE),[12] polyacrylamide gel electrophoresis (PAGE),[10,16] denaturing gradient gel electrophoresis (DGGE),[17] single-strand conformation polymorphism analysis,[18] and, most recently, temperature gradient gel electrophoresis.[19]

Slack et al[20] recently described the amplification of TCR-γ gene rearrangements (TCR-γGR) from paraffin-embedded biopsies with consensus primers for the most frequently used TCR-γ genes. Lorenzen[21] described a protocol for paraffin tissue with primers for all variable region genes and a consensus joining region primer.

With an adequate detection method, small biopsies from tissue such as punch biopsies of skin, colon biopsies, and transbronchial biopsies could be approached by PCR. An added benefit of the ability to amplify gene rearrangements from paraffin-embedded tissues is the reduction in cost achieved by sparing the patient an additional surgical procedure. An additional procedure also leads to further delay in diagnosis.

DGGE has been most frequently used for mutation detection for genetic diseases[22,23] and the characterization of restriction fragment length polymorphisms. DGGE, single-strand conformation polymorphism analysis, and temperature gradient gel electrophoresis are powerful techniques that separate segments of DNA based upon the nucleotide sequence and not merely the length of the DNA, the limiting characteristic of routine AGGE and PAGE. In single-strand conformation polymorphism analysis, the PCR-amplified DNA is heat denatured into single-stranded DNA and subsequently electrophoresed through a nondenaturing polyacrylamide gel.[24] In DGGE, the PCR-amplified double-stranded DNA is electrophoresed through a denaturing gradient of urea and formamide.[23] The addition of guanine- and cytosine-rich sequences (so-called GC clamps) to the 5′ end of one of the primers has been shown to increase the resolution of DGGE for detecting mutations in genetic diseases.[23] Sheffield et al[25] and Abrams et al[26] have described the capability of separating DNA sequences with single nucleotide changes with GC-clamped DGGE.

Bourguin and co-workers[17] were the first to describe the use of a DGGE system to detect clonal TCR-γGR. In their PCR technique, they used consensus primers to amplify only the group I TCR Vγ genes and several Jγ genes. PCR products were concentrated into a smaller volume after ethanol precipitation and analyzed on a vertical 6.5% polyacrylamide gel with a 30 to 60% denaturing gradient. Clonal TCR-γGR were identified by the presence of a predominant band(s) on the gel, whereas a polyclonal population was assumed when only a smear was obtained. Resolution capabilities of specific TCR-γ DNA sequences were not demonstrated nor was DGGE applied to paraffin tissues.

The purpose of this report is to present a modified DGGE protocol incorporating a 40-nucleotide (nt) GC clamp into the PCR products for the detection of known combinations of clonal TCR-γGR. We show that the incorporation of a GC clamp into TCR-γ PCR products enhances the separation of a clonal TCR-γGR from background polyclonal TCR-γGR compared with routine electrophoretic methods. Using this modified DGGE protocol, we also show increased resolution over non-GC-clamped DGGE to demonstrate clonal bands with similar DNA sequences. We also show that it can be effectively applied to archival tissue.

Materials and Methods

DNA from 5 well characterized T cell lines (Jurkat, Molt-4, HSB2, 8402, and CEM), a peripheral T cell lymphoma, and an alloreactive T cell clone, all with known TCR-γGR, were used to establish the efficacy of the primers in DGGE. In addition, 2 B cell lines (Raji

and H937), 7 mononuclear cell samples from normal individuals, and 3 cases/cell lines of Hodgkin's disease, all which lacked TCR-γGR,[3] were analyzed as negative controls. In addition, DNA from 9 cases of acute lymphoblastic leukemia from the University of Iowa, 10 cases of acute lymphoblastic leukemia from the National Cancer Institute, and 27 cases of lymphoblastic lymphoma from the National Cancer Institute were studied. Lymphoblastic leukemia/lymphoma (LL) was chosen to illustrate the DGGE protocol, as all T cell LL and a high percentage of B cell LL exhibit TCR-γGR. In addition, lysates from paraffin-embedded tissue of 20 T cell lymphomas characterized by immunophenotyping and 5 paraffin-embedded reactive lymph nodes were also studied.

DNA was obtained from fresh tissue by proteinase K digestion, phenol/chloroform extraction, and ethanol precipitation as described.[27] Southern blots were performed on the 5 T cell lines, 2 B cell lines, the 3 Hodgkin's cases, and 12 cases of LL. DNA (10 μg) was digested with *Eco*R1, *Kpn*I, or *Bam*HI, Southern blotted as described,[28] and hybridized with the M13H60 Jγ probe provided by J. Sklar (Stanford University) or the Jγ probe provided by Tak W. Mak (University of Toronto).[2]

For paraffin-embedded tissues, DNA was obtained from 1 cm^2 of 6- to 10-μ sections that were cleared of paraffin with xylene, washed with ethanol, and dried in a 50 C block. The tissue was digested in buffer with 10 mmol/L Tris-HCl (pH 8.3), 50 mmol/L KCl, 0.5 mg/ml proteinase K for 30 minutes at 50 C after sonication for 10 minutes.[29] Alternatively, it was incubated overnight at 50 C. The samples were heated to 95 C for 10 minutes to inactivate proteinase K and centrifuged. In the PCR amplification, 1 to 10 μl of lysate was tested for β-globin amplification of 268-bp products with primers PC04 and GH20.[30] If successful, PCR of TCR-γGR was pursued.

Individual primers (Table 1) were synthesized via a solid phase triester method on a Dupont synthesizer for each of the four main groups (I to IV)[9,31–34] of variable (Vγ) region genes, which contain Vγ1–8, Vγ9, Vγ10, and Vγ11 genes, respectively. The Vγ primer sequences are located approximately 206, 130, 110, and 90 nt, respectively, from the TCR junctional region (Figure 1). In addition, primers were chosen for the joining region (Jγ) genes Jγ1/Jγ2, Jγp, Jγp1, and Jγp2, with the 5′ end of each located about 50 to 55 nt from the junction (Figure 1). A 40-nt GC clamp,[35] which does not anneal to target sequences in the genome, was synthesized at the 5′ end of the Vγ primers. A GC-clamped Jγ2 primer was used with non-clamped Vγ primers in limited comparative studies of the 10 sequenced ALL DNA samples. For sensitivity studies, serial dilutions were performed with HSB2 DNA in placental DNA and in a second experiment in peripheral blood lymphocyte DNA. A GC-clamped Vγ9 primer and the mixture of Jγ primers described above were used in the sensitivity studies.

For the PCR, genomic DNA (0.5 to 0.6 μg) or lysate (1 to 10 μl) was added to PCR buffer (10 mmol/L Tris-HCl (pH 8.3), 1.5 mmol/L $MgCl_2$, 50 mmol/L KCl, and 0.01% gelatin) 100 μmol/L dNTPs, 2.5 U *Taq* polymerase enzyme (Perkin-Elmer, Norwalk, CT) added at 94 C, 1 μmol/L of the selected GC-clamped Vγ primer, and 0.6 μmol/L of each of the four Jγ primers in a total volume of 100 μl overlaid with 50 μl of light mineral oil (Sigma Chemical Co., St. Louis, MO). Ten DNA samples previously sequenced from 9 patients were also amplified with a GC-clamped Jγ primer paired

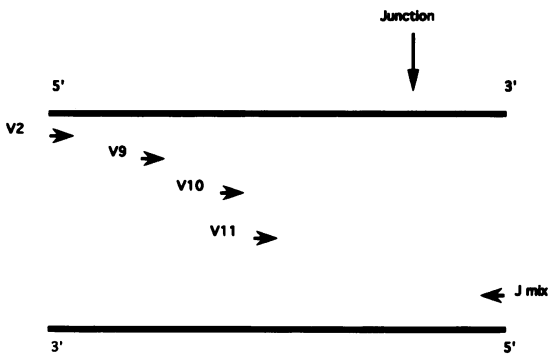

Figure 1. *Relative locations of the four Vγ primers and the Jγ mix of four primers from the junctional area of the TCR-γGR.*

Table 1. *Sequences of the Primers Used in the Polymerase Chain Reaction Protocol*

Primer	Sequence 5′-3′	Reference
Vγ2	TAC ATC CAC TGG TAC CTA CAC CAG	31
Vγ9	GAA AGG AAT CTG GCA TTC CGT CAG	9,32
Vγ10	AAG CAA CAA AGT GGA GGC AAG AAA G	33,34
Vγ11	AGT AAA AAT GCT CAC ACT TCC ACT TC	33
Jγ2	TAC CTG TGA CAA CAA GTG TTG TTC	31
Jγp	AAG CTT TGT TCC GGG ACC AAA TAC	32
Jγp1	GAA GTT ACT ATG AGC TTA GTC CCT T	33
Jγp2	GAA GTT ACT ATG AGC CTA GTC CCT T	33
GC clamp	CGC CCG CCG CGC CCC GCG CCC GGC CCG CCG CCC CCG CCC G	35

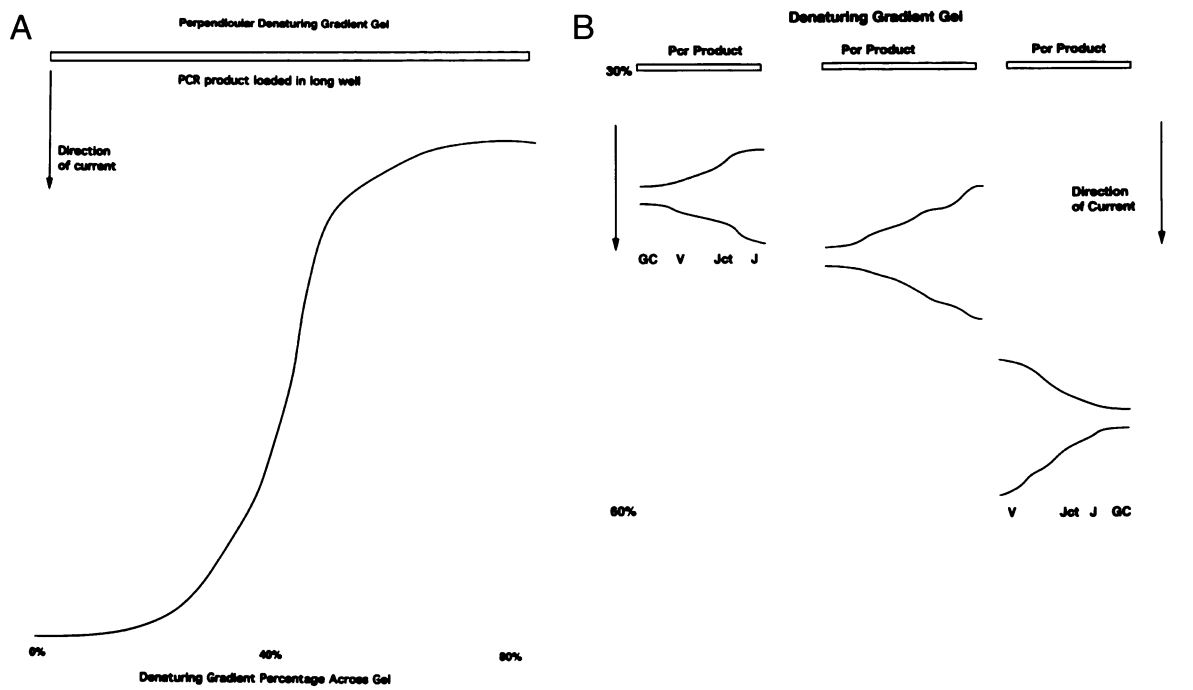

Figure 2. A: *Perpendicular denaturing gradient gel. The electrophoretic current travels vertically through the gel but the gradient extends horizontally (perpendicular) ranging from 0 to 80% denaturing. The midpoint of the S curve determines the center of the gradient range bracketed in subsequent parallel gels. In this example of Vγ9 GC it is approximately 45%.* B: *Parallel denaturing gradient gel. The electrophoretic current travels vertically in the gel in the same direction as the increasing denaturing gradient. PCR products partially separate and slow in mobility at the melting temperature corresponding to the unique junctional sequence.*

with a Vγ2 primer without a GC clamp. To avoid contamination, solutions were transferred with fiber-plugged pipette tips, gloves were changed frequently while handling PCR products, and PCR reactions were set up and products amplified in separate rooms.

After an initial denaturation at 94 C for 9 minutes, PCR was performed on a Model 480 Thermocycler (Perkin-Elmer) with 40 cycles of denaturation at 94 C (75 seconds), annealing at 66 C (75 seconds), and extension at 72 C (1 second with automatic extension of 1 second per cycle). A final extension at 72 C was performed for 7 minutes. Before DGGE, 10 μl of each PCR product were screened by AGGE in 3.0% agarose (GIBCO BRL, Gaithersburg, MD) with 100 mmol/L Tris, 90 mmol/L boric acid, and 1 mmol/L EDTA, pH 8.3, buffer.

A total of 80 μl of PCR reactions with identifiable products on AGGE were dried on a heated Model 100 DNA vacuum concentrator (Savant Instruments, Farmingdale, NY). The dried products were resuspended in 2 μl of sterile deionized water and combined with 8 μl of a loading solution (20% sucrose, 10 mmol/L Tris-HCl, pH 7.8, 1 mmol/L EDTA, and 0.1% bromphenol blue.)

For DGGE, 15-cm × 20-cm × 0.75-mm gels with a 30 to 60% denaturing gradient range were poured with 8% polyacrylamide (acrylamide:bis-acrylamide at 37:1) in 40 mmol/L Tris, 40 mmol/L sodium acetate, 1 mmol/L EDTA, pH 7.4 (1X TAE), mixed with 8% polyacrylamide in 1X TAE with 5.6 mol/L urea and 32% formamide. These two polyacrylamide stock solutions have a denaturing effect of 0 and 80%, respectively. The catalyst concentrations of 0.023% TEMED and 0.09% ammonium persulfate were chosen to harden the gel after 30 minutes, allowing time to pour the 23-ml gradient gels with a gradient maker (GIBCO BRL). The DGGE system (CBS Scientific Co, Del Mar, CA) contained 27 liters of 1X TAE at pH 7.4 heated to 60 C.

The optimal range of the denaturing gradient in the analytic parallel gels was first determined by analyzing TCR-γGR products in a perpendicular gel with a 0 to 80% range (Figure 2A). The denaturing gradient used in the analytical DGGE was constructed vertically in the gel so the electrophoretic current travels in parallel with the gradient (Figure 2B). The denaturing midpoint was found to be approximately 45%; therefore, a denaturing range of 30 to 60% was used in analytical parallel gels.

Results

Definitions and Sensitivity

A positive rearrangement of one or two alleles on DGGE was defined as the presence of one to two prominent bands that were significantly more intense than the background smear. Rarely, three or four bands were observed secondary to the formation of heteroduplexes between the two rearranged alleles. The observed PCR product lengths with the GC clamps were approximately 300 nt for Vγ2, 220 nt for Vγ9, 200 nt for Vγ10, and 180 nt for Vγ11. A polyclonal population was represented by a broad smear in the gel.

The expected Vγ and Jγ genes were amplified in each of the five T cell lines and 12 LL containing known TCR-γGR gene rearrangements as determined by traditional Southern blot analysis (data not shown). The sensitivity of the GC-clamped DGGE was 0.1% when HSB2 DNA was serially diluted in placental DNA (Figure 3), but a lower level of sensitivity (1%) was observed when diluting in polyclonal T cell DNA (data not shown).

Comparison of DGGE with and without GC Clamps

A comparative analysis demonstrates the higher resolution of rearranged bands obtained by GC clamp incorporation in the modified DGGE. Clearly delineated bands are seen in each ALL sample with the Vγ2GC product (Figure 4, lanes 6 to 8), but the result for ALL 290 without the GC clamp in lane 3 is difficult to interpret.

Correlation with TCR-γGR Sequences

In each case with a TCR-γGR, a unique migration pattern was observed despite similarities in the length

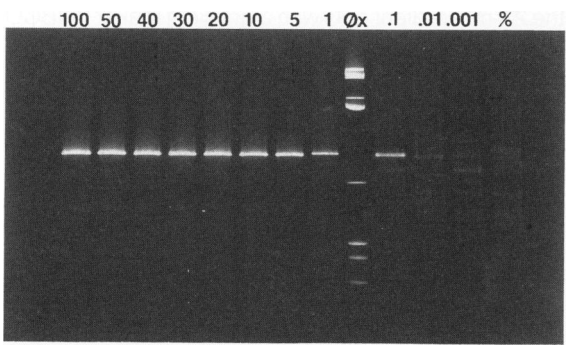

Figure 3. *Parallel gel of T cell clone HSB2 DNA with Vγ9 rearrangement serially diluted in placental DNA. Percentage HSB2 DNA is indicated. PCR in a total of 1 μg of DNA with Vγ9 GC clamp and Jγ primer mix.*

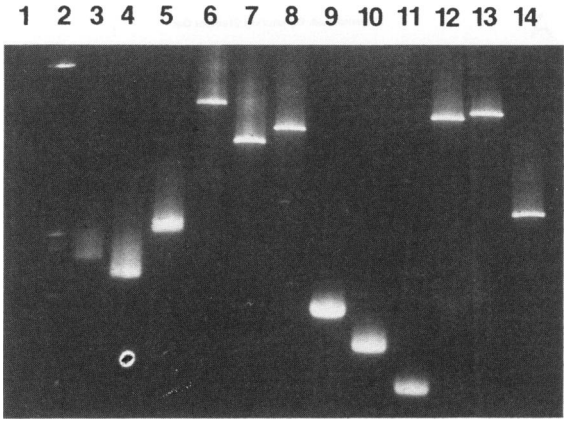

Figure 4. *Parallel gel with 30 to 60% denaturing gradient comparing DGGE with and without GC-clamped primers.* Lane 1, *negative control;* lane 2, *ϕX DNA marker;* lanes 3 *to* 5, *Vγ2, ALL 290, ALL 306, ALL 769;* lanes 6 *to* 8, *Vγ2GC, ALL 290, ALL 306, ALL 769;* lanes 9 *to* 11, *Vγ9, LL 2252, HSB2, LL1709; and* lanes 12 *to* 14, *Vγ9GC, LL 2252, HSB2, LL1709.*

of the PCR product (Figure 5). The TCR-γGR of 10 rearrangements of 9 cases of ALL were found to contain group I Vγ genes rearranged to Jγ1 or Jγ2. Table 2 contains the V genes and the net size of the PCR products of the 10 TCR-γGR rearrangements. The 4 ALL cases with Vγ4 genes (Figure 5, lanes 3 to 6) illustrate the power of GC-clamped DGGE to separate PCR products based on sequence. There is a strikingly large separation between ALL 467 (lane 5) and ALL 306 (lane 6), although both Vγ4 products differ by only 5 bp. The longest PCR product, 308 bp, in ALL 283 (lane 3), migrates farther than ALL 467, which is 10 bp shorter. This electrophoretic pattern is the opposite of what is expected in routine AGGE and PAGE, both of which separate double-stranded DNA only on the basis of length. A similar difference in electrophoresis is seen between ALL 001 (lane 10), with

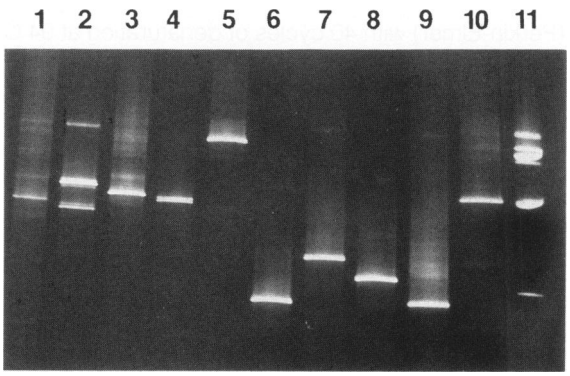

Figure 5. *Parallel gel with a 30 to 60% denaturing gradient. PCR with Vγ2 GC clamp and Jγ primer mix amplifying 10 TCR-γGR. Lane 11 is ϕX DNA marker.* Lane 1: *ALL 139, 302 bp (net +2); V2 gene.* Lane 2: *ALL 355, 296 bp (−4); V2.* Lane 3: *ALL 283, 308 bp (+8); V4.* Lane 4: *ALL 493, 300 bp (0); V4.* Lane 5: *ALL 467, 298 bp (−2); V4.* Lane 6: *ALL 306, 293 bp (−7); V4.* Lane 7: *ALL 290, 286 bp (−14); V2.* Lane 8: *ALL 769, 302 bp (+2); V3.* Lane 9: *ALL 229, 299 bp (−1); V8.* Lane 10: *ALL 001, 264 bp (−36); V7.*

Table 2. *Junctional Sequences from 11TCR-γGR in Nine Cases of ALL and One Case of Peripheral T Cell Lymphoma (549)*

	DNA	Gene	PCR product	Vγ*	N†	Jγ‡
1	283§	Vγ4	308 bp	TGGGATGG	AAGGAGGGC	GAATTATTATAAGAA
2	290§	Vγ2	286 bp	TGGGA	G	AGAA
3	467§	Vγ4	298 bp	TGGGA	CAA	AATTATTATAAGAA
4	229	Vγ8	299 bp	TGGGATA	CTGTTTCTCG	TAAGAA
5	139	Vγ2	302 bp	TGGGACGGG	AA	GAAGTATTATAAGAA
6	493	Vγ4	300 bp	TGGGATG	CTTCC	TTATTATAAGAA
7	355	Vγ2	296 bp	TGGGAC	TC	TTATTATAAGAA
8	769	Vγ3	302 bp	TGGGACAGG	CCGG	ATTATTATAAGAA
9a	001	Vγ7	264 bp		CCCCTGGG	AAGAA
9b	306	Vγ4	293 bp	TGGGATGGG	CCCTG	GAA
10	549‖	Vγ2	303 bp	TGGGACGGG	AAA	GAAGTATTATAAGAA

*Shows the nt remaining in the 3′ Vγ gene segment after deletion has occurred. A 34 nt deletion occurred in the V7 region of case 9a.
†Shows the nt inserted in the N region that are not of germline origin.
‡Shows the nt remaining in the 5′ Jγ gene segment after deletion has occurred.
§These cases have also had archival bone marrow smears amplified with non-GC-clamped primers, yielding the same sequence.
‖The same sequence was identified in serial biopsies from 1988, 1989, and 1990. DGGE demonstrated an identical band in biopsies from 1988, 1989, 1990, and 1991.

a Vγ7 product of 264 bp, and ALL 229 (lane 9), with a Vγ8 product of 299 bp. In this pair the migration is affected by the different Vγ gene sequences used as well as by the unique junctional sequences.

A comparative study is demonstrated between agarose gels (Figure 6) and DGGE analysis (Figure 7). In agarose the electrophoretic migration is compressed because it is dependent solely on the length of the PCR products.

Effect of the Location of the GC Clamp

Better separation of the TCR-γGR bands was obtained by DGGE with the GC-clamped Vγ primers compared with the GC-clamped Jγ primer (Figure 7). The theoretical basis for this phenomenon is based on the distance of the GC clamp from the unique sequences present in the junction of the TCR-γGR. With the GC clamp located at the 5′ end of the Vγ primer, the clamp is 90 to 200 nt away from the junctional region. Less separation is seen with the GC clamp located at the Jγ 5′ end (Figure 7), which is only 50 nt away from the junctional region (Figures 1 and 2B). To optimize the separation of similar sized DNA, the junctional region should be located in the melting region distant from the GC clamp.

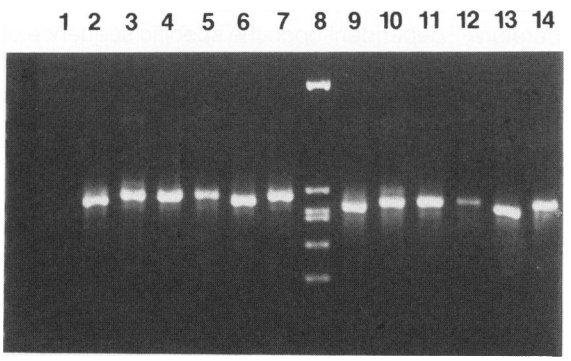

Figure 6. *A 3.0% agarose gel of PCR with Vγ2 GC clamp and Jγ2 primers in* lanes 1 *to* 7. *PCR with Jγ2 GC clamp and Vγ2 primers in* lanes 9 *to* 14. Lane 1, *negative control (no template DNA);* lanes 2 *and* 9, *ALL 290;* lanes 3 *and* 10, *ALL 355;* lanes 4 *and* 11, *ALL 467;* lanes 5 *and* 12, *ALL 493;* lanes 6 *and* 13, *ALL 306;* lanes 7 *and* 14, *ALL 769; and* lane 8, *ϕX DNA marker.*

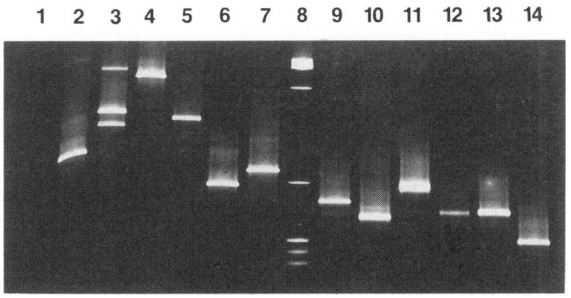

Figure 7. *Parallel gel with a 30 to 60% denaturing gradient. PCR with Vγ2 GC clamp and Jγ2 primers in* lanes 1 *to* 7. *PCR with Jγ2 GC clamp and Vγ2 primers in* lanes 9 *to* 14. Lane 1, *negative control;* lanes 2 *and* 9, *ALL 290;* lanes 3 *and* 10, *ALL 355;* lanes 4 *and* 11, *ALL 467;* lanes 5 *and* 12, *ALL 493;* lanes 6 *and* 13, *ALL 306;* lanes 7 *and* 14, *ALL 769; and* lane 8, *ϕX DNA marker.*

Efficacy in Polyclonal T Cell Populations

Multiple bands may be observed with TCR-γ probes on Southern blots of polyclonal T cells,[3] confounding the detection of true rearrangements. No clonal bands were identified by DGGE in the two B cell lines, seven mononuclear cell samples from normal individuals, or the three cases/cell lines of Hodgkin's disease analyzed (Figure 8). The seven polyclonal T cell and three Hodgkin's disease samples were previously reported to contain a ladder of bands on TCR-γ Southern blot analyses, the expected finding in polyclonal T cells.[3] On DGGE analysis a polyclonal smear was obtained in each case, indicating the lack of a predominant clonal TCR-γGR.

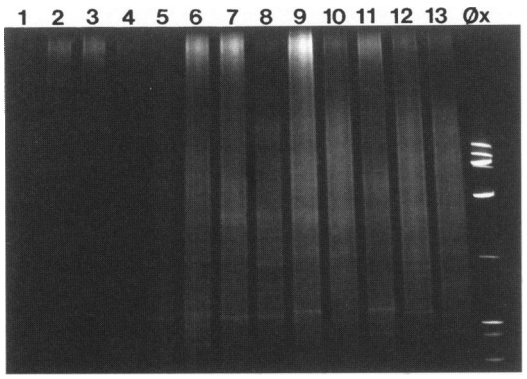

Figure 8. *Parallel gel with a 30 to 60% denaturing gradient. PCR with Vγ2 GC clamp, Vγ9 GC clamp primers and Jγ primer mix.* Lane 1, *negative control;* lane 2, *Raji,* lane 3, *U927;* lane 4, *L428;* lanes 5 *to* 11, *normal peripheral blood lymphocytes;* lanes 12 *and* 13, *Hodgkin's disease; and ϕX DNA marker. No predominant bands are present.*

Lymphoblastic Lymphoma/Leukemia

Of the 46 LL cases, 42 exhibited TCR-γGR on DGGE analysis. The 12 LL containing known TCR-γGR, determined by traditional Southern blot analysis, revealed 100% concordance with positive DGGE results for the specific Vγ genes used in the TCR-γGR. Of the remaining 34 LL cases studied by DGGE alone, 30 were positive for TCR-γGR.

Paraffin-Embedded Tissues

In the paraffin-embedded tissues, 17 of 20 T cell lymphomas had clonal rearrangements identified by DGGE (Figure 9), 2 were indeterminate for the presence of a rearrangement and 1 was negative (Table 3). In 6 cases with both fresh tissue DNA and paraffin-embedded tissue available, identical rearrangements were seen in the archival and fresh DNA samples. These include peripheral T cell lymphoma[1] (Figure 9) lymphoblastic lymphoma (1), γδ T cell lymphoma (2), and malignant T cell lymphoma not otherwise subclassified (2).

Discussion

The Southern blot analysis of TCR-γGR has not been widely used due to the difficulties that have been encountered with the interpretation of multiple bands often observed with DNA derived from polyclonal T cells.[3] In Southern blot analysis the electrophoretic separation in agarose is based solely on the length of DNA fragments produced by the restriction enzymes. We believe that the genetic information resident within TCR-γ genes is useful. Whereas Southern blotting may be an inadequate tool to extract this information for TCR-γGR, GC-clamped DGGE is a powerful tool as the separation of the PCR products is based on the unique DNA sequences present in the rearranged gene and not just the length of the DNA.

Normal peripheral blood T lymphocytes contain a diversity of polyclonal TCR-γGR secondary to the multiplicity of immune responses to exogenous antigens. Examination of the junctional sequence of the polyclonal TCR-γGR present has revealed that there is a relatively small variation in the length of the rearranged genes, but significant diversity is present in the junction between the variable (V) and joining (J) genes. Tamura et al[36] described the Vγ9 TCR-γGR mRNA transcripts in normal peripheral blood lymphocytes. If Vγ9 and Jγ primers are chosen to amplify a 200-nt sequence around the published sequences of the Vγ9-Jγ junctions,[36] a relatively narrow distribution in length of the products would be observed (Figure 10). Deletions of the Vγ and Jγ sequences along with insertions at the junction provide the diversity for TCR rearrangements. One can easily deduce why routine agarose gel electrophoresis or PAGE may have difficulty in separating a clonal band from a polyclonal smear in low level disease. The majority of the rearrangements are of similar size ±20 nt in normal lymphocytes[36] or ALLs.

Each DNA sequence has a different melting temperature[22] dependent upon the specific sequence of the TCR-γGR. The GC clamp, by allowing for partial

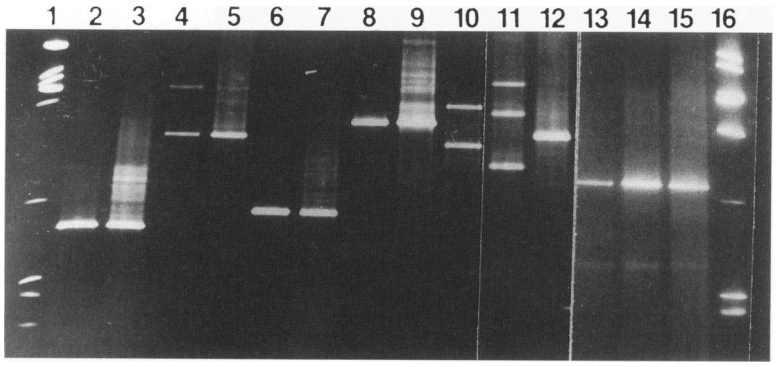

Figure 9. *Parallel gel with 30 to 60% gradient of paraffin-embedded cases of T cell lymphomas.* Lane 1 *ϕX DNA marker;* lanes 2 *and* 3, *mycosis fungoides case 1;* lanes 4 *and* 5, *mycosis fungoides, case 2;* lanes 6 *and* 7, *large cell;* lanes 8 *and* 9, *γδ T cell;* lane 10, *subcutaneous T cell 1;* lane 11, *subcutaneous T cell 2;* lane 12, *malignant lymphoma NOS;* lanes 13 *to* 15, *peripheral T cell lymphoma with* lane 13 *containing 1988 fresh tissue DNA 549,* lane 14 *1988 paraffin tissue and* lane 15, *1990 paraffin tissue; and* lane 16, *ϕX DNA marker for* lanes 13 *to* 15. Lanes 3, 5, 7, *and* 9 *represent double round PCR products.*

Table 3. *T Cell Gene Rearrangements Identified in Paraffin-Embedded Tissue*

Diagnosis	Result			
	Positive*	Negative	Indeterminate	Total
Peripheral T cell	1	0	0	1
Mycosis fungoides	2	0	0	2
Large cell immunoblastic	2	0	0	2
γ/δ	2	0	0	2
Diffuse mixed small and large cell	1	0	0	1
Malignant T cell, NOS	4	1	1	6
Lymphoblastic	1	0	0	1
Subcutaneous T cell	4	1	0	5
Reactive hyperplasia	0	5	0	5

*Positive prominent bands of TCR-γ rearrangements are identified. Negative, no prominent bands are seen. Indeterminate, numerous faint bands are present in a polyclonal smear.

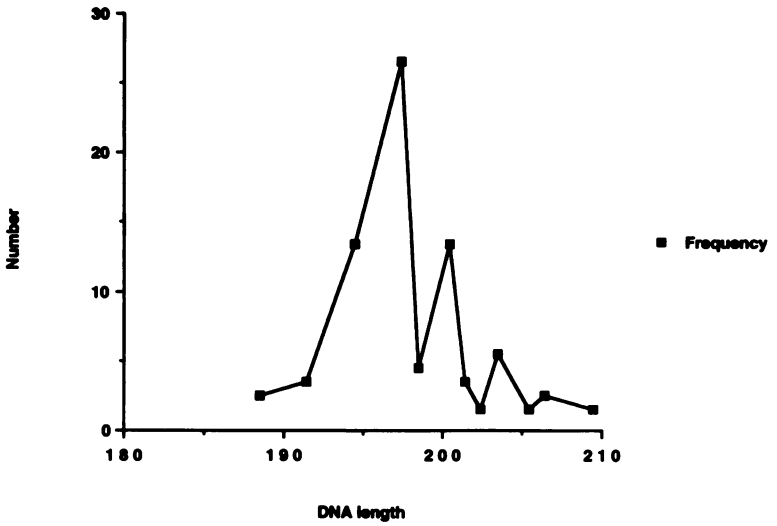

Figure 10. *Frequency distribution of the calculated lengths of PCR products from Tamura et al[36] if primers were designed to amplify 200-bp products of TCR-γGR.*

denaturing of the PCR product as it electrophoreses through the denaturing gel, forces the separation of the PCR products to be influenced by the unique sequence of the junction, strategically located near the 3′ end (Figure 2). As the 5′ sequence in the five ALL cases known to contain the Vγ4 gene is the same until the junction (Table 1), variability in the melting of the DNA segment is dependent on the variable portion of the junction. These results confirm that the migration is dependent on the tumor-specific rearranged junctional TCR-γGR sequence.

The GC-clamped DGGE method provides a mechanism for internal quality control to detect contamination that cannot be done well with routine electrophoretic methods. Short of sequencing or restriction enzyme digests of the PCR product, one cannot determine whether an amplified band is due to the patient under study or contamination from a previous PCR reaction. The need for developing quality control mechanisms for identifying TCR-γGR by PCR analysis has been vividly demonstrated by the retraction of Imrie et al[37] who discovered that six of the eight positive samples in a previous report[10] had the same TCR-γGR upon sequencing the PCR products. Because their negative control showed no evidence of contamination, and because TCR-γGR vary little in length, there was no easy way to determine that all six of the PCR products contained the same TCR-γGR on routine electrophoresis. The problem of contamination, also observed by Slack,[20] has been inadequately addressed in previous papers on detecting TCR-γGR by PCR and is pertinent as laboratories consider applying PCR protocols on clinical cases.

We demonstrated excellent correlation between DGGE and Southern blot analysis. The correlation extended to the identification of the specific Vγ gene amplified in the PCR, which was the same Vγ gene(s) as that determined by Southern blots of *Eco*R1 or *Kpn*I digests. In addition, we demonstrated the same rearrangement in paraffin material as in fresh tissue DNA.

Our report demonstrates that a nonradioactive method can be used to detect clonal TCR-γGR with greater sensitivity and accuracy than routine AGGE or

PAGE. This approach also avoids the potential problem of not being able to detect contamination that may occur in the DNA samples by previously amplified PCR products. The addition of the 40-nt GC clamp provides better delineation of the bands by generating a DGGE DNA fingerprint of the clonal TCR-γGR than DGGE without GC clamps. We have correlated the DGGE analysis with the resolution of sequences of the TCR-γ rearrangements. GC-clamped DGGE provides a nearly unique signature for each clonal sequence. Therefore, repeated finding of PCR products with the same DGGE mobility should alert the investigator or clinical pathologist to possible PCR contamination.

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