To detect occult lymphoma, the polymerase chain reaction (PCR) technique was used to amplify joined bcl-2/JH DNA sequences at the junction of the \( t(14;18) \) translocation in follicular lymphoma. Using the heat-stable DNA polymerase \( \text{Taq} \) and automated cycling of the reaction, we were able to detect as few as one to two copies of bcl-2/JH. Under these conditions, PCR proved to be at least 10,000-fold more sensitive than either conventional flow cytometry or Southern blot restriction analysis. In addition, genomic DNA sequences of four lymphomas confirmed that the size of the amplified segment serves as a tumor marker. Direct application of PCR to patient staging revealed occult malignant lymphomas in tissue otherwise considered uninvolved by standard criteria. We conclude that the striking enhancement in diagnostic sensitivity attained by DNA amplification can serve as a valuable adjunct to the staging and clinical monitoring of follicular lymphoma.

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**PROCEDURES**

*Patient samples.* High molecular weight (mol wt) DNA was extracted from solid tissue and peripheral blood lymphocytes, and from cells of the SUDHL-6 B-cell lymphoma line using previously described methods. Approval was obtained from the Institutional Review Board for the studies described. Patients were informed that tissue samples might have been obtained for research purposes but that their privacy would be protected and any results that might impact on their medical care would be communicated to their physicians.

**DNA amplification.** Amplifications were performed both manually and with an automated method. The manual procedure was carried out in a 50-\( \mu \)L volume with \( \text{Taq} \) DNA polymerase (New England Biolabs, Beverly, MA) in a thermostable buffer containing 33 \( \mu \)mol/L each of dATP, dTTP, dCTP, and dGTP in accordance with the manufacturer’s recommendations. In addition, 1 \( \mu \)g of each primer and a range of 1 pg to 2.4 \( \mu \)g of starting genomic DNA were included. The following primers were added to each reaction: 5'-TTAGAGAGTTGCTTTACGTCGGCTG-3' (complimentary to the negative strand segment 5' of the major breakpoint region), 5'-AAACAAAAAGCCTAGGTCGGCCATT-3' (complimentary to the positive strand of JH6), 5'-GGACTACCTGAGGA-GACGGTGACC-3' (complimentary to the positive strand containing a conserved sequence of JH1, JH2, JH4, and JH5), and 5'-CATTTCACCTGAGACAGCGTGACC-3' (complimentary to the positive strand of JH3). One hundred nanomoles of mineral oil were layered over the reaction mixture prior to initiation of the PCR. To denature the genomic DNA, samples were placed in a 95°C water bath for seven minutes. Primers were annealed to their complimentary single genomic strands in a 23°C bath for five minutes. Two units of \( \text{Taq} \) polymerase were then added, and the samples were incubated in a water bath at 63°C for five minutes to extend the annealed primers. In subsequent cycles, denaturation was carried out at 91°C for one minute followed by annealing in a 23°C bath for one minute and then primer extension at 63°C for five minutes. Two units of \( \text{Taq} \) DNA polymerase were added after the initial 20 cycles, and 20 additional rounds of polymerization were performed.

Automated PCR amplification was performed with a Thermocycler and \( \text{Taq} \) polymerase (Perkin-Elmer/Cetus, Norwalk, CT) in accordance with the instructions of the manufacturer while using the

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LYMPHOMA DETECTED BY PCR

above primers. The reaction mixture, containing from 10 pg to 10 ng genomic DNA, was heated to 94°C for seven minutes, cooled to 55°C for five minutes, and brought to 74°C for five minutes. Following this initial round, samples were heated at 91°C, 55°C, and 74°C for 1.5, 1.5, and 5 minutes, respectively, for a total of 40 cycles.

Southern blot analysis. Twenty microliters of each PCR-amplified sample were resolved by electrophoresis (80 V, five hours) in 1.5% agarose gels with 0.5 μg ethidium bromide/mL in tris-borate buffer. Alkaline Southern transfers were performed overnight using Genescreen Plus nylon filters (DuPont, NEN Products, Boston) and 0.4 N NaOH as a transfer buffer. Blots were probed with a 32P-labeled 2.8-kb EcoRI/HindIII fragment of the bcl-2 major breakpoint region and a 32P-labeled genomic JH SauIIIA fragment and autoradiographed, as described.4 To confirm that sequences detected were generated from primer-directed amplification, blots were reprobed with 5'-CAACACAGACCCACCCAGAGCCCTCTGCCCCTTCCTGGCGGGG-3', the sequence of which is complimentary to a genomic region immediately 3' to the bcl-2 primer.11,13

Conventional restriction fragment analysis of genomic DNA was performed by endonuclease digestion with EcoRI, BamHI, and HindIII, electrophoretic separation of the restriction fragments, and Southern transfer, as described.4 Blots were hybridized with 32P-labeled genomic probes of JH, J-κ, C-λ, and bcl-2 (2.8-kb EcoRI-HindIII fragment).4

RESULTS AND DISCUSSION

The sensitivity of the PCR technique was investigated using serial dilutions of DNA extracted from SU-DHL6, a B-cell lymphoma line that carries the t(14;18) translocation characteristic of follicular lymphoma. Using both the manual and automated procedures, a 250-bp DNA sequence was amplified, which hybridized with genomic probes for both bcl-2 and JH and with the internal oligonucleotide probe (Fig 1A). This indicates that the amplified segment contained the DNA sequence at the point of juncture of chromosomes 14 and 18. The length of this amplified segment is precisely that which is predicted from the known DNA bcl-2/JH sequence of SU-DHL6.12 The amplified bcl-2/JH sequence was detectable even when as little as 10 pg of SU-DHL6 genomic DNA was tested by the automated method or with 100 pg using the manual technique (Fig 1A).

The amount of DNA detected with the automated procedure represents the DNA equivalent of less than two cells.
Because the reaction can be conveniently carried out with as much as 2.5 μg of starting genomic DNA (3.75 × 10^6 cells), the potential sensitivity of detection is 1.0 cell/2 × 10^6.

Next, we addressed the specificity of the PCR/bcl-2 technique as a tumor marker. DNA from three cases of follicular lymphoma (patients 1003, 1032, and 1144) and from the SU-DHL6 cell line was amplified. Because the exact location of the breakpoint within the major breakpoint cluster region of chromosome 18 and the JH region involved differs slightly from case to case, different sized fragments should be generated by PCR. The sizes of the amplification products generated (Fig 1B) corresponded exactly to the sizes predicted by the known DNA sequences of each of these samples (Fig 1C). As a control, DNA extracted from four normal peripheral blood lymphocyte samples, three T-cell lymphomas, and human placenta was also subjected to the PCR, and no signal was detected (data not shown).

Because the size of the amplified sequence varies among lymphoma samples, the specific size of the generated fragment can serve as an individual tumor marker. This feature could prove useful for monitoring patients to identify second neoplasms and to assist in the determination of relapse by detection of residual disease following therapy. To assess this, we sought to apply the PCR approach retrospectively to samples obtained from patients who have received treatment for follicular lymphoma.

Patient 1029 presented with follicular lymphoma involving an inguinal lymph node. The initial biopsy was performed at another institution and fresh tissue was not available for further studies. A second enlarged axillary lymph node was diagnosed as normal by conventional histology, and immunophenotyping using flow cytometry revealed 70% T cells and polyclonal B cells (16% Igκ, 10% Igλ). Therefore, by standard criteria, the lymphoma was stage I at presentation, and local radiation therapy was delivered to the inguinal and iliac lymph node regions. A clinical remission was achieved and maintained for 21 months, at which time an enlarged submandibular lymph node became apparent. Histologic examination of the submandibular lymph node demonstrated follicular lymphoma, mixed small and large cell type. DNA obtained from the histologically uninvolved axillary lymph node biopsied at presentation and from the submandibular lymph node was subjected to both restriction fragment analysis and PCR. Immunoglobulin heavy and light chain gene rearrangements and a bcl-2 rearrangement indicative of a monoclonal B-cell lymphoma were readily detected in the relapse specimen (Fig 2A, lane b), but no rearrangements were seen in the pretreatment, histologically uninvolved axillary lymph node (Fig 2A, lane a). By contrast, PCR generated a clearly identifiable bcl-2/JH segment from both specimens. This fragment was the same size in each sample (Fig 2B), indicating that lymphoma cells existed in the histologically uninvolved lymph node and were clonally related to the lymphoma at relapse. Thus, our study has confirmed that PCR can be applied to directly demonstrate the presence of occult disease and might be used to predict relapse where other approaches fail.

It is of note that the diagnostic sensitivity of PCR as shown here is in part attributable to enhancement of both the specificity and sensitivity inherent in the use of the Taq polymerase in comparison with the less sensitive system using the Klenow fragment of DNA polymerase.14 The thermostable DNA polymerase purified from the Thermus aquaticus bacterium, Taq, can survive extended incubation at 95°C, allowing annealing and extension of the primers at more stringent, higher temperatures, which results in greater specificity than that achieved with conventional DNA polymerase. Also, in contrast to Klenow, Taq is unaffected by the heat denaturation step and does not need to be added with each amplification cycle. This feature greatly simplifies the PCR procedure and permits the use of automation. Moreover, the automated technique demonstrated a level of sensitivity to the manual approach, presumably as a consequence of consistency of temperature regulation in a single, controlled oil bath as opposed to repeated transfers among three water baths for the manual method.

In summary, the PCR technique can be applied to detect the joined bcl-2/JH sequence of follicular lymphoma and to provide a unique tumor marker to diagnose, stage, and monitor the course of disease. The sensitivity of the method has permitted the specific demonstration of lymphoma cells that were sufficiently rare to elude detection by standard
morphology or sensitive immunophenotyping and genotyping techniques. The PCR approach might prove useful for resolving clinical questions regarding relapse in follicular lymphoma. The ability to identify occult disease following treatment might afford earlier and more accurate assessment of the effectiveness of therapy. Not only could this information contribute to the management of individual patients, but it could accelerate the pace of clinical trials through the demonstration of resistant tumor before frank clinical relapse occurs. In addition, PCR analysis of purged bone marrow harvested for autologous transplant of lymphoma patients could serve to prevent reinfusion of malignant lymphoma cells. However, caution must be exercised in the interpretation of a positive finding, since we do not yet know if such a minor population of cells will proliferate. Longitudinal clinical trials must be performed to determine the clinical significance of occult lymphoma before management decisions can be made on the basis of PCR analysis.

REFERENCES

Detection of occult follicular lymphoma by specific DNA amplification

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