Cytometric Detection of DNA Amplified With Fluorescent Primers: Applications to Analysis of Clonal bcl-2 and IgH Gene Rearrangements in Malignant Lymphomas

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Follicular lymphomas comprise almost two thirds of the US adult non-Hodgkin's lymphomas (NHL) and are the most common malignancy of B-lineage lymphocytes. Polymerase chain reaction (PCR) protocols have been developed to detect the t(14;18) translocation, which juxtaposes the bcl-2 proto-oncogene to the Ig heavy-chain (IgH) gene in 85% of follicular lymphomas and monoclonal rearrangements of the IgH gene in B-cell NHL that lack bcl-2 rearrangements. We used PCR to amplify bcl-2 and IgH rearrangements in DNA from patients with lymphoproliferative disorders and analyzed the products in parallel by gel electrophoresis and flow cytometry, which detected PCR products incorporating fluoresceinated oligonucleotide primers by sequence-specific capture to oligonucleotide-coated magnetic beads. Overall, flow cytometry was superior to electrophoresis of ethidium-bromide-stained agarose gels for detection of products of nested PCR to detect intergenic rearrangements involving bcl-2 and single primer-pair amplification of clonal rearrangement of IgH. Flow cytometric analysis detected bcl-2 translocations in 12 of 13 CD10+ B-cell lymphomas and clonal IgH rearrangements in 14 of 17 monoclonal B-cell populations. In contrast, analysis by gel electrophoresis detected bcl-2 translocations in only 10 of 13 CD10+ and clonal IgH gene rearrangements in only 9 of 17 monoclonal B-cell populations. Flow cytometric analysis was more sensitive than gel electrophoresis and could detect a 16-fold greater dilution of a bcl-2-amplified product than gel electrophoresis. Similarly, flow cytometry could detect an amplification product when template DNA was diluted 10,000-fold, whereas gel electrophoresis only detected amplification products when template was subjected to dilution between 100- and 1,000-fold. This shows the utility of flow cytometry for the analysis of DNA amplification products incorporating fluorochrome-labeled primers as a rapid, objective alternative to conventional strategies. Because current-generation clinical laboratories emphasize automation, flow cytometric analysis of PCR-amplified products shows increased analytic sensitivity and offers a vehicle for automation of DNA amplification tests.

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rescinated primers by flow cytometry from patients with lymphoproliferative disorders, compare these results with those obtained by standard molecular biology techniques, and discuss its possible clinical applications.

**MATERIALS AND METHODS**

**Samples.** Eighteen specimens consisting of bone marrow (BM), lymph node (LN), peripheral blood (PB), or other tissue from 17 patients sent to our flow cytometry laboratory for routine immunophenotyping were obtained for use in this study. The tissue type, immunophenotypic diagnosis, and CD10 positivity, a cell surface marker detectable in 75% to 80% of follicular lymphomas,13 given for each sample in Table 1. The study was designed to include a majority of CD10+ specimens to increase the likelihood that these samples contained bcl-2 gene rearrangements. Many of the CD10+ specimens were selected in an initial screening in a bcl-2 PCR analyzed by gel electrophoresis; therefore, the percentage of bcl-2 translocation positive follicular lymphomas reported in this study does not represent the actual percentage of bcl-2 translocation positive follicular lymphomas in our general patient population.

**DNA extraction.** Genomic DNA from all samples and controls was extracted using a 341 Nucleic Acid Purification System (Applied Biosystems, Foster City, CA) according to protocols supplied by the manufacturer. Typically 1 to 3 mL of BM or PB or 1 g of LN or other tissue was loaded onto the instrument for DNA extraction. The precipitated DNA was dissolved in H2O and stored at −20°C.

**Oligonucleotide synthesis.** All oligonucleotides used in this study were synthesized using a 391 DNA synthesizer (Applied Biosystems) using reagents and protocols supplied by the manufacturer. Biotin or fluorescent dye were added to the 5' end of certain oligonucleotides were desalted using a Sephadex G-25 (Sigma). Alternatively, fluorescent dye phosphoramidite (FAM) were puriﬁed using Oligonucleotide Purification Cartridges (Applied Biosystems) and protocols supplied by the manufacturer. All other oligonucleotides were desalted using a Sephadex G-25 (Sigma Chemical Co, St Louis, MO) column.

**Coupling of capture oligonucleotide to beads.** Seventeen micro-liter (approximately 10^12) of streptavidin-coated super paramagnetic polystyrene beads (2.8 μm in diameter; Dynal, Inc, Great Neck, NY) was washed twice in 20 μL phosphate-buffered saline to remove sodium chloride, once in 20 μL of 1× binding and washing (B&W) buffer (5 mmol/L Tris-HCl pH 7.5, 0.5 mmol/L EDTA and 1.0 mmol/L NaCl) separated with a magnetic separation stand (Promega Corp, Madison, WI), and resuspended in 50 μL 1× B&W buffer. Sixteen and five tenths picomoles of the bcl-2 PCR capture oligonucleotide (5' biotin-GTGTTGAAACAGGCCACGTAA-AAGCAACTCT 3') or the IgH capture oligonucleotide (5' biotin-CTGGGGCCAAGNACCTGTGT 3') was added to the beads and the volume adjusted to 100 μL with 1× or 2× B&W buffer for a final NaCl concentration of 1.0 mol/L. The beads were incubated for 30 minutes at room temperature, washed twice in 100 μL 1× B&W buffer, once in hybridization buffer (HB; 6× SSPE [900 mmol/L NaCl, 60 mmol/L NaOH, 50 mmol/L EDTA, pH 7.4], 0.1% sodium dodecyl sulfate), and resuspended in 80 μL HB. The beads were incubated at room temperature for 30 minutes after addition of 20 μL of denatured salmon testes DNA (1 μg/μL) in HB.5 The beads were washed twice in 100 μL HB, resuspended in 200 μL HB, and stored at 4°C.

**bcl-2/MBR PCR.** Each sample was amplified by nested PCR, ie, the original amplified product was reamplified by a second PCR, using a second set of oligonucleotide primers internal to those primers used in the original PCR.3

PCR consisted of a 100-μL final volume using 0.5 μg of sample DNA, 1 μmol/L of oligonucleotide primers, 200 μmol/L each of deoxy adenosine triphosphate, deoxy cytidine triphosphate, deoxy guanosine triphosphate, and deoxy thymidine triphosphate, and 1.5 U Taq DNA polymerase (Promega Corp) in PCR buffer (500 mmol/L KCl/100 mmol/L Tris-HCl pH 9.0, 1.0% Triton X-100, 1.5 mmol/L MgCl2). Before addition of sample DNA, PCR mixes were irradiated with 254 nm and 366 nm UV for 5 to 10 minutes to inactivate contaminating DNA. The first amplification was performed in a 9600 thermal cycler (Perkin-Elmer Cetus, Norwalk, CT) for 30 cycles using the oligonucleotide primers 5' CCAAGTCATGTTGACATTCCAGC 3' and 5' ACCCTGAGAGACGGTGACTACC 3'. Each cycle consisted of three 30-second steps: 94°C denaturation, 57°C annealing, and 72°C extension. A 5-μL aliquot of the first PCR was used as the DNA template for the 30 cycle second, or nested, round of PCR using the oligonucleotide primers 5' FAM-TATGTTGTTGGACCTTATTAG 3' and 5' ACCAGGTTCCCTTGGCCCCA 3' under the same conditions as the first amplification. These nested primers should amplify a product of approximately 140 to 250 bp. Four plasmid DNA templates (0.5 μg of each) were used as normal controls and 5-μL of R. cell line DNA, which contains a PCR-amplifiable breakpoint at the MBR, was used as a positive control. The R.L. cell line was kindly provided by J.G. Gribben (Dana-Farber Cancer Institute, Harvard Medical School, Boston, MA). Negative controls containing no template DNA were used to assess contamination, and β-globin PCR was performed on each sample to ensure that the extracted DNA was suitable for amplification. Amplification, all reactions were dried in a vacuum centrifuge.

**IgH gene PCR.** Each sample was amplified for 40 cycles using the oligonucleotide primers 5' ACACGGCC(C/T) (G/C)TGTATTGGGGCCAAGNACCCTGGT 3'.3 Each cycle consisted of three 30-second steps: 94°C denaturation, 60°C annealing, and 72°C extension. DNA from a B-cell NHL, 0.5 μg, was used as a positive control. All other conditions were identical to those used in the bcl-2/MBR PCR.

**Gel analysis.** One set of each bcl-2 and IgH PCR was analyzed by electrophoresis in a 2% agarose gel (SeaKem LE agarose; FMC Bioproducts, Rockland, ME) containing tris-acetate buffer and ethidium bromide; although the fluorescent PCR products can be visualized with UV without addition of ethidium bromide to the gel (albeit faintly), addition of ethidium bromide greatly increases the intensity of the amplified products and is necessary to visualize the nonfluorescent molecular weight markers. Amplification reactions containing a discrete band at the expected electrophoretic migration were considered to be positive. The amplified DNA was visualized by ethidium bromide staining and photographed on a Faxitron gel documentation system (Faxitron, Barksdale, TX). Negative controls containing no template DNA were used to assess contamination, and β-globin PCR was performed on each sample to ensure that the extracted DNA was suitable for amplification. Amplification, all reactions were dried in a vacuum centrifuge.

**Hybridization and flow cytometric analysis.** A second set of bcl-2 and IgH PCR products was resuspended in 10 μL H2O after concentration, to which 9 μL of 2× HB were added. Each sample was then denatured at 94°C for 2 minutes in a 9600 thermal cycler followed by a 30-minute 42°C hybridization. (The amplified DNA may be heat denatured at end of PCR and quench cooled at 4°C before concentration, if desired.) Avidin-coated beads (5 × 10^9 beads in 1 μL) coated with the biotinylated allele-specific capture oligonucleotide, bcl-2 or IgH, were added to each tube after the thermal cycler had reached 42°C. Preliminary hybridization experiments (data not shown) indicated that use of a single oligonucleotide designed to capture one strand of the PCR product resulted in a stronger positive signal than using two oligonucleotides designed to capture both strands of the amplified product. After hybridization, the beads were washed three times with buffer (100 mmol/L Tris pH 8.5, 10 mmol/L EDTA, 50 mmol/L NaCl, 0.01% NP-40, and 0.01% Tween 20) at room temperature for at least 30 seconds. The beads were resuspended in 100 μL sheath fluid (Couler Corp, Hialeah, FL) and analyzed for fluorescence using an Epics Elite
Flow Cytometer (Coulter) using standard protocols for fluorescein. MCF was determined for each sample. Samples whose values were above a 99% confidence interval constructed from the normal controls using Student's t distribution were considered to be positive.

RESULTS

Flow cytometric analysis of bcl-2 and IgH PCR-amplified DNA is superior to gel electrophoresis. A comparative analysis of bcl-2 and IgH PCR-amplified DNA by flow cytometry and gel electrophoresis is shown in Table 1. For each specimen and control, the MCF and the ratio of the specimen MCF to the normal control mean MCF(S/C) are listed from the flow cytometric analysis and the intensity or absence of a UV-visualized ethidium-bromide-stained band is listed from the gel-electrophoresis analysis (Table 1).

Immunophenotyping showed that 13 of the 18 specimens were CD10⁺ (Table 1). Eleven of these specimens were diagnosed as follicular small cleaved cell lymphoma and the other two (specimens 12, 18) as diffuse small cleaved cell NHL. Gel electrophoresis of bcl-2 PCR-amplified DNA detected 10 of the 13 CD10⁺ lymphomas and a CD10⁻ diffuse small cleaved cell lymphoma (specimen 15). Flow cytometry detected bcl-2 rearrangements in 12 of the 13 CD10⁺ lymphomas, as well as the diffuse small cleaved cell lymphoma, also evident by gel electrophoresis. Specimen 12, positive for bcl-2 translocation by gel electrophoresis, was negative by flow cytometry. This may be caused by specimen contamination or within the bcl-2 capture oligonucleotide sequence that would preclude its capture.
Fluorescence electrophoresis of DNA amplified using the IgH gene consensus primers detected 9 of the 17 monoclonal populations, but also detected a monoclonal population in specimen 6 that was diagnosed as a polyclonal B-cell proliferation because there were proportionate subpopulations that expressed \( \kappa \) - or \( \lambda \) - light chains by immunophenotyping. Presumably, this represents a false positive, perhaps caused by a prominence of an individual B-lymphocyte specificity. Flow cytometry detected a monoclonal population in 14 of the 17 samples, but did not detect a significant amplification product in specimen 6, from the patient with a polyclonal hyperplasia. Specimen 17 was positive by gel electrophoresis for IgH rearrangement, but was negative by flow cytometry, possibly because of sequence heterogeneity in the amplified segment of the IgH gene not represented in the capture oligonucleotide designed from consensus J-region sequences. However, six specimens (3, 7 through 9, 12 and 13) positive by flow cytometry were negative by gel electrophoresis. MCF for positive samples ranged from 8.5 (specimen 5) to 4.9 (specimen 15), whereas corresponding S/C ranged from 2.4 to 1.4.

Overall, analysis of \( bcl-2 \) and IgH PCR-amplified DNA by flow cytometry of fluorescent PCR product captured by oligonucleotide-bound magnetic beads was superior to gel electrophoresis of PCR-amplified DNA. Flow cytometric analysis detected 12 of 13 CD10\(^+\) \( bcl-2 \) translocations and 14 of 17 monoclonal B-cell populations without any false positives; gel electrophoresis analysis detected 10 of 13 CD10\(^+\) \( bcl-2 \) translocations and only 9 of 17 monoclonal B-cell populations with one presumably false positive. Flow cytometry readily distinguished DNA containing \( bcl-2 \) and monoclonal IgH gene rearrangements from normal DNA (Fig 1). The capture of PCR products by oligonucleotide-bound beads is very specific and sequence dependent, because PCR products did not bind to salmon testes DNA-treated beads in the absence of the allele-specific capture oligonucleotides. Moreover, the amplification product generated from templates in the IgH positive control (B-cell

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**Fig 1. Comparison of \( bcl-2 \) and IgH-positive samples to normal controls.** (A) \( bcl-2 \). Specimen 4 is represented by the solid lines and normal control 3 by the stippled lines. (B) IgH. Specimen 5 is represented by the solid lines and normal control 3 by the stippled lines. The horizontal axis represents fluorescence intensity (arbitrary units) and the vertical axis represents number of events (paramagnetic beads).
Flow cytometric analysis of bcl-2 PCR-amplified DNA is more sensitive than gel electrophoresis. To determine whether flow cytometric analysis was more sensitive than gel electrophoresis, fourfold serial dilutions were made of bcl-2 PCR-amplification products from bcl-2 translocation-positive (specimen 4) and bcl-2 translocation-negative (normal control 3) templates. The PCR product dilutions were analyzed in parallel by flow cytometry (Fig 2) and gel electrophoresis (Fig 3). Flow cytometric analysis confidently detected a 1:1024 dilution of the bcl-2 PCR product, whereas gel electrophoresis could not detect the 248-bp positive product below a 1:64 dilution with confidence.

Next, 10-fold dilutions were made of the same template DNAs that were then amplified in a PCR containing primers to detect amplification of bcl-2 rearrangement. Portions were analyzed by flow cytometry and gel electrophoresis. Flow cytometry was able to detect a 10,000-fold dilution of the bcl-2 positive template (Fig 4), whereas gel electrophoresis only detected a dilution between 1:100- and 1:1,000-fold of the same template after PCR (data not shown).

**DISCUSSION**

Amplification of clonal rearrangements involving the bcl-2 and/or IgH genes is an effective adjuvant for the diagnosis and staging of B-cell NHL. However, this technique has not been widely applied, perhaps, in part, because of the lack of a facile strategy for analysis of amplifications reactions. We describe a rapid, objective approach for analysis of DNA amplification reactions that is more sensitive than standard methods and that lends itself to automation and multiparameter detections of products of multiplex PCR reactions.

Crescenzi et al detected the bcl-2/MBR translocation by PCR in 100% (7 of 7) of patients with follicular lymphoma, and Gribben et al detected this translocation by nested PCR in 88% (22 of 25) of patients with follicular lymphoma. These results are similar to our data, where 11 of 11 samples diagnosed as follicular lymphoma were positive for the bcl-2/MBR translocation by flow cytometry analysis of PCR-amplified DNA using bcl-2/MBR primers and IgH gene consensus primers; analysis by gel electrophoresis detected the translocation in 9 of 11 cases.

Crescenzi et al reported the ability to amplify a single template copy of the bcl-2 breakpoint and a limit of sensitivity of one tumor cell in 10⁶ normal cells for Southern blotting and nucleic acid hybridization of bcl-2 PCR-amplified DNA. Gribben et al reported a similar sensitivity for gel electrophoresis of nested bcl-2 PCR products. Our inability to detect bcl-2 PCR-amplification products by gel electrophoresis from specimen 4 templates diluted between 1:100- and 1:1,000-fold suggests that at least approximately 70 to 700 copies of template are needed to detect bcl-2 amplification products by gel electrophoresis in our hands. (These calculations are based on 6 pg DNA/cell and specimen 4 consisting of approximately 80% tumor cells.) This decrease in sensitivity is caused by our treatment of PCR mixes with UV to inactivate any contaminating DNA that may be present. A 1,000-fold decrease in sensitivity has been reported...
gel electrophoresis analysis of the cases by gel electrophoresis. Gel electrophoresis of IgH template PCR; this level of sensitivity is not necessary for most of the lymphomas submitted to serial dilutions of tumor cell in 10^6 normal cells (data not shown). However, previously for UV treatment of certain PCR mixes. When we do not irradiate PCR mixes with UV, the sensitivity of gel electrophoresis analysis of the bcl-2 PCR increases to one tumor cell in 10^6 normal cells (data now shown). However, this level of sensitivity is not necessary for most of the lymphomas submitted to us for routine evaluation, and we prefer to use UV irradiation of PCR mixes to eliminate any DNA contamination, unless it is necessary to do otherwise.

Trainor et al detected monoclonal IgH gene rearrangement by PCR gel electrophoresis in 83% (19 of 23) of cases of B-cell NHL or B-cell CLL. We detected B-cell monoclonality in 14 of 17 (82%) of samples by flow cytometry analysis of PCR-amplified DNA and 9 of 17 (53%) of cases by gel electrophoresis. Gel electrophoresis of IgH PCR-amplified products detects only monoclonal proliferations that constitute at least 2% to 5% in a background of a reactive, polyclonal population. Indeed, in three of the specimens that were negative by gel electrophoresis but positive when analyzed by flow cytometry, the content of neoplastic monoclonal B-lymphocytes was below the limits of resolution described for analysis of ethidium-bromide-stained gels. Thus our rate of detection of clonal IgH rearrangements by electrophoretic analysis of PCR is not dissimilar to that described by others. The increased sensitivity of flow cytometric analysis of fluorescent amplification products is evident from the detection of IgH gene amplification in the same three cases. Moreover, because amplification of polyclonal rearrangements may result in faint smearing, visual interpretations of IgH PCR reactions may be ambiguous and fraught with pitfalls. The alternative approach of flow cytometry, which is quantitative, mitigates this shortcoming at least in part.

Our modification of the PCR-IRB assay to directly couple fluorescein to a PCR oligonucleotide primer during its synthesis so that one strand of the final PCR product, itself, contains fluorescein, obviates the use of digoxigenin-11-dUTP and FITC-conjugated antibody. We have also reduced the number of magnetic beads used in each hybridization (5 x 10^5 v 4 x 10^5 for the PCR-IRB) and have used shorter capture oligonucleotides (30 mer for bcl-2 and 21 mer for IgH v a 42 mer for the hepatitis B PCR-IRB) with a lower hybridization temperature (42°C v 60°C for hepatitis B PCR-IRB) that still resulted in sequence-specific PCR product capture.

The PCR-IRB assay was able to detect hepatitis B DNA in 5 x 10^{-10} and lower dilutions of plasma. This method of detecting amplified DNA by flow cytometry was 10-100-fold more sensitive than slot-blot hybridization of PCR products using radioisotope-labeled probe. Part of this increased sensitivity was caused by the initial use of hepatitis B antibody-coated magnetic beads to isolate the virus from infected plasma in the PCR-IRB. This technique was not applicable to our study. Regardless, the highest S/C reported in the PCR-IRB study was 4.6. Our highest S/C for bcl-2 PCR was 31.3 and for IgH PCR was 2.4.

Another advantage of flow cytometric analysis of PCR products is its objectivity. Cut-off points can be objectively defined using statistical techniques. Yang et al used seven times the SD of the mean of their negative control as the cut-off of PCR-IRB positivity. We found that a 99% confidence interval of the normal controls (~3 SD) best suited our data. Gel electrophoresis analysis may not be as objective. Although in most cases of nested bcl-2 PCR, a prominent band is present or absent, in IgH PCR, it is sometimes difficult to distinguish a distinct band from a small "smear" (a "smear" not being indicative of a monoclonal B-cell population). In the current study, such an occurrence resulted in a false positive in gel electrophoresis analysis. Moreover, because flow cytometric analysis is 10-100-fold more sensitive than gel electrophoresis in detecting products of single primer pair and nested PCR, it may be possible to run fewer amplification cycles, thereby lowering the risk of contamination.

Although conventional PCR with isotopic nucleic acid hybridization is considered the "gold standard," Gribben et al reported that the use of radiolabeled internal oligonucleotide probes did not result in any further increase in the sensitivity of the nested bcl-2 PCR, compared with analysis by gel electrophoresis alone. Our findings indicate that flow cytometric analysis of PCR-amplification products may have significant advantages over other approaches; however, in some contexts, the determination of the product size by electrophoresis may provide worthwhile information. Overall, flow cytometric analysis of fluorescein-containing PCR products captured with oligonucleotide-coated magnetic beads is more sensitive than PCR analysis by gel electrophoresis, and its use may be more amenable to routine clinical flow cytometry laboratories than standard molecular biol-
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ogy techniques. Because current-generation flow cytometers emphasize automated sample handling, this alternative, which promises increased analytic sensitivity and simultaneous analysis of multiple amplification products, also offers capability of automation of DNA amplification tests in lymphoproliferative and other disorders.

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Cytometric detection of DNA amplified with fluorescent primers: applications to analysis of clonal bcl-2 and IgH gene rearrangements in malignant lymphomas

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