

Sensitivity of PCR in detecting monoclonal B cell proliferations

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Abstract

Aims—To evaluate the rapid detection of various forms of monoclonal B cell proliferations by using the polymerase chain reaction (PCR) to identify clonal immunoglobulin heavy chain genomic rearrangements.

Methods—Thirty four B cell lymphomas defined by morphology, immunophenotyping, and positive immunoglobulin heavy chain gene rearrangements detected by Southern blot analysis were examined. An additional 22 cases representing miscellaneous lymphoproliferative and non-lymphoproliferative disorders were also studied.

Results—Monoclonal rearrangements were identified in 19 (56%) cases of B cell lymphoma. The method was less sensitive in the detection of follicular centre cell lymphomas (15 of 28, or 54%) than non-follicular centre cell lesions (four of six, or 67%). Monoclonal rearrangement was not identified in 19 control cases, including T cell lymphomas, Hodgkin's disease, reactive lymphadenopathy and metastatic carcinoma. Three cases showed positive immunoglobulin gene rearrangement by PCR but were negative on Southern blotting. Two of these cases had definite clinical, morphological, and immunophenotypic evidence of monoclonal B cell proliferation suggesting that PCR could, on occasion, pick up cases missed by Southern blotting and that the two methods are complementary in clonal lymphoproliferative disease diagnosis. The third case represented a "false positive" PCR reaction involving a colonic adenocarcinoma.

Conclusions—PCR analysis, using the primer sequences outlined in this study, will detect about 55% of clonal lymphoproliferative proliferations with increased sensitivity for non-follicular centre cell lesions. With these levels of detection in mind, this testing strategy can still be especially useful in cases which prove diagnostically problematic with standard morphological and immunophenotypic analysis, and in instances where the quantity and type of diagnostic material is limiting (needle aspirates and cellular fluids).

With the advent of the cloning and characterisation of the immunoglobulin heavy and light chain genes has come the possibility of a more sensitive approach in identifying monoclonal B cell proliferations.^{1,2} Gene rearrangement studies by Southern blotting using immunoglobulin heavy chain (IgH) and light chain gene probes provide evidence of clonality and cell lineage with a sensitivity unmatched by routine histology and immunophenotyping. DNA analysis can detect monoclonal B cell proliferations even when neoplastic cells represent only 2-5% of a mixed cell population.³

Genetic analysis using conventional Southern blotting, however, has some major disadvantages. The technique is expensive and complex, requires the use of radioactive isotopes, and the reporting turnaround time, of at least one to two weeks, greatly decreases its value in contributing to initial patient management. Two groups have recently and independently described a new strategy using PCR to amplify the V-D-J region of the IgH gene for the differentiation of monoclonal from polyclonal B cell proliferations.^{4,5} Both groups used a single V region primer specific for sequences present in most V region genes, corresponding to the third framework region (Fr3). The results of their initial studies compared favourably with the IgH gene rearrangement study by Southern blot analysis. The simplicity and speed (results available in 24 hours) of the PCR technique offers major advantages over Southern blotting as a diagnostic aid in the routine laboratory.

To evaluate the application of the PCR technique in the routine diagnostic workup of a variety of lymphoproliferative disorders, a study was undertaken to investigate the sensitivity and specificity of this method in the detection of B cell non-Hodgkin's lymphoma. The PCR results were correlated with the findings of histology, immunophenotyping, and Southern blotting gene rearrangement studies.

Methods

A study was made of 56 cases for which DNA from either fresh or freshly frozen tissue was obtained. The samples included 36 lymph node biopsy specimens, 15 bone marrow aspirates, and five biopsy specimens or excision of lesions in the lung (two), spleen (one), brain (one) and colon (one). All cases

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Accepted for publication
9 February 1993

had initially been studied and classified as B or T non-Hodgkin's lymphoma, Hodgkin's disease, reactive lymphadenopathy, or metastatic carcinoma by routine histological examination immunophenotyping (by either flow cytometry or immunohistochemical stains on frozen sections, or both), and Southern blot analysis of the IgH and T cell receptor β genes. Thirty four cases were B cell lymphomas with monoclonal IgH gene rearrangement on Southern blotting. Of these 28 were follicular centre cell lymphomas⁶ and the remaining six cases were small lymphocytic lymphomas. Two additional cases had features of B cell lymphoma on histological examination and immunophenotyping, but were negative on Southern blot analysis of the IgH gene. The remaining 20 cases included reactive lymphadenopathy (five), reactive marrow lymphocytosis (seven), T cell lymphoma (two), Hodgkin's disease (three), metastatic carcinoma (two), and colonic carcinoma (one).

DNA was extracted from either fresh or freshly frozen tissue by treatment with sodium dodecyl sulphate-proteinase K, followed by phenol-chloroform extraction, as described before.⁷ The DNA extracted was precipitated with two volumes of ethanol and redissolved in 1mM TRIS-10mM EDTA buffer.

DNA was digested with the restriction endonucleases *EcoRI*, *HindIII*, and *BamHI*. The digested DNA was size fractionated by agarose gel electrophoresis and then transferred to a nylon membrane (Gene Screen Plus, Dupont Canada Inc.) by Southern blotting.⁸ The membrane was hybridised with an α -³²P-dCTP labelled probe from the J region of the IgH gene.¹ After washing to a stringency of 0.1 x standard saline citrate, 0.1% sodium dodecyl sulphate at 65°C for 30 minutes, the blot was subjected to autoradiography for one to seven days.

The PCR was performed according to the method described by Trainor and Wan^{4,9} with some modifications. The primers used were: for the third framework portion of the V region, 5' ACACGGC(C/T)(G/C)TGTAT-TACTGT 3' (Fr3A); for the J region, 5' TGAGGAGACGGTGACC 3' (LJH), or 5'

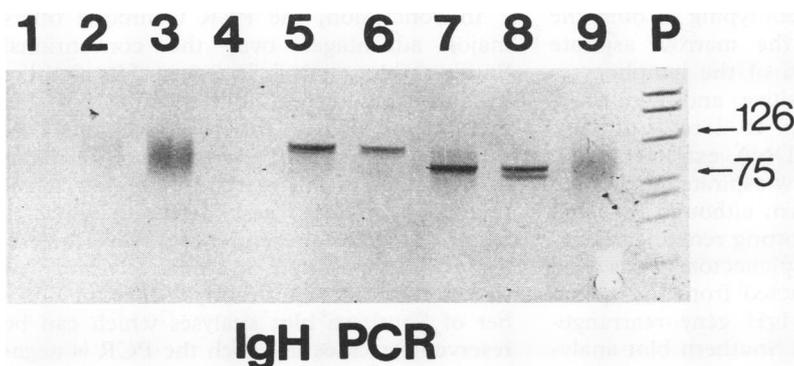
GTGACCAGGGTNCCTTGGCCCCAG 3' (VLJH). A "nested" PCR was performed in a Perkin-Elmer Cetus Thermal Cycler using *Thermus aquaticus* (*Taq*) polymerase (Perkin-Elmer Cetus). The PCR reaction mixture (100 μ l) contained 0.2 μ M of each primer, 5 units of *Taq* polymerase, 0.8 mM of a dNTP mix, 3mM magnesium sulphate, 16.6 mM ammonium sulphate, 10 mM β mercaptoethanol, and 67 mM TRIS-HCl (pH 8.0). A first round of 25 PCR cycles was performed using primers Fr3A and LJH. In the second round of 20 PCR cycles Fr3A and the internal nested primer VLJH were used. Each PCR cycle consisted of denaturation at 94°C for 2 minutes, annealing at 60°C for 2 minutes, and extension at 72°C for 2 minutes. The final amplified product was electrophoresed on a 12% polyacrylamide gel and visualised under ultraviolet light after staining with ethidium bromide. Stringent laboratory guidelines were followed during the entire procedure to avoid DNA cross contamination and PCR product carry-over.¹⁰ Tubes with no DNA template were included as negative controls in each run.

Results

Of the 34 cases of B cell non-Hodgkin's lymphomas defined by morphology, immunophenotyping, and clonal IgH gene rearrangement on Southern blotting, only 19 showed monoclonal rearrangements on PCR (sensitivity of 56%). The rate of positive PCR was lower in the follicular centre cell group (15 of 28, or 54%) than in non-follicular centre cell lesions (four of six, or 67%). The two cases that were morphologically and immunophenotypically consistent with B cell lymphoma but were negative for IgH rearrangement on Southern blotting both showed positive results by the PCR technique. No monoclonal rearrangement pattern was seen in 19 of the 20 control cases, including T cell lymphomas, Hodgkin's disease, reactive lymphadenopathy and metastatic carcinoma (specificity of 95%). A single false positive PCR result was observed in a case of colonic carcinoma. Examples of the PCR findings are shown in the figure.

Discussion

The detection of monoclonal rearrangement of the IgH gene by the PCR technique provides a simple and rapid method for the differentiation of monoclonal from polyclonal B lymphocyte proliferations. Using this technique, Trainor *et al* successfully identified 19 of 23 (83%) cases of B cell lymphoma/leukaemia.⁴ McCarthy *et al*, using a different set of V-J primers in the PCR, detected eight of 10 (80%) cases of B cell lymphoma.⁵ With increasing experience in the use of the PCR technique, however, McCarthy recently reported a decrease in their positive detection rate from the initially described 80% to 62% (16 of 26 cases).¹¹ The test was highly specific and no false positive reactions were found by either group of investigators.



IgH PCR

PCR analysis of IgH gene rearrangement. Results of 12% polyacrylamide gel electrophoresis after PCR amplification of DNA from five cases of B cell lymphoma (tracks 4, 5, 6, 7 and 8), two cases of reactive lymphadenopathy (tracks 3 and 9), and one case of T cell lymphoma (track 2). Track 1 is a negative control in which no DNA template was included in the reaction. Track P is marker DNA with relevant size markers labelled in base pairs along the right hand side.

Our study showed positive PCR results in 19 of the 34 B cell lymphomas studied, translating to a sensitivity of only 56% in lymphoma detection. This result is more in keeping with the current experience of McCarthy *et al* than with the initial reports which claimed a detection rate of 80%. Possible reasons for a failure of amplification in some cases include suboptimal priming by currently available primers which are designed from a limited number of published human immunoglobulin variable region genes, and the presence of mutation or translocation involving the IgH gene.

The finding of a lower detection rate in follicular centre cell lymphoma is particularly interesting. There is accumulating evidence to suggest that somatic hypermutation of the immunoglobulin gene variable regions occurs predominantly in follicular centre cells during antigen driven B cell proliferation.^{12,13} Furthermore, continuous somatic mutation in the immunoglobulin gene is known to occur frequently in follicular centre cell lymphoma.¹⁴ A reciprocal translocation between chromosomes 14 and 18 is a common finding in follicular centre cell lymphoma. t(14;18) is detected by cytogenetics in about 85% of follicular small cleaved cell lymphomas, while 20% of diffuse large cell lymphomas show molecular evidence of this translocation.¹⁵ This translocation brings the proto-oncogene BCL2 into the IgH locus and may affect the priming and amplification of the IgH gene by the PCR procedure. Thus somatic hypermutation and molecular rearrangements unique to follicular centre cell lymphomas may be the underlying cause for the difficulty in this PCR amplification. It is therefore important for subsequent studies to specify the yield of the PCR analysis according to different lymphoma types so that this can be verified.

Two cases of B cell lymphoma, defined by characteristic morphological, immunophenotypic, and clinical findings, were found to be positive on PCR but were negative on Southern blotting. The first case involved a 59 year old woman who presented with acquired C1 esterase inhibitor deficiency and splenomegaly. Bone marrow examination showed patchy infiltration by small cleaved lymphocytes consistent with metastasis by lymphoma. Immunophenotyping of both the peripheral blood and the marrow aspirate showed that 35 to 40% of the lymphocytes were B cells (CD 19 positive) and there was λ light chain restriction (κ to λ ratio of 1:6). PCR amplification of DNA extracted from both blood and a marrow aspirate yielded the same monoclonal pattern, although repeated analysis by Southern blotting remained negative. Subsequently, a splenectomy was performed and DNA extracted from the spleen showed a monoclonal IgH gene rearrangement on both PCR and Southern blot analysis. The second case involved a 76 year old woman with a monoclonal gammopathy (free κ light chains in the urine) and infiltration of the bone marrow by a mixture of lymphocytes and atypical plasma cells consistent with

marrow disease as a result of lymphoplasmacytic lymphoma. Repeated analysis of the IgH locus by Southern blotting was negative while PCR showed a distinct monoclonal pattern.

It is important to realise that although the sensitivity for case detection of B cell lymphoma by the new PCR method is much lower than that of Southern blot analysis, because of the inherent technical problems involved in IgH gene amplification, the sensitivity of detecting monoclonal B cells in a mixed lymphocyte population by PCR is similar to Southern blotting in cases in which IgH genes can be amplified. Both methods can detect a population of 2–5% of monoclonal B cells.^{4,5} The above two cases also show that there are instances in which the PCR method may outperform Southern blot analysis by detecting monoclonality missed by the latter method, and for these reasons we regard the two methods as being complementary in the diagnosis of lymphoma. PCR analysis has two additional advantages over Southern blotting in relation to the types of diagnostic material that can be studied. A PCR strategy can be used on DNA extracted from paraffin wax embedded fixed tissue, a substrate that rarely yields DNA of sufficient quality for Southern analysis. PCR can also be used to good effect on diagnostic samples such as needle aspirates and cellular fluids which can be limiting both in terms of the amount and quality of cells available for study.

Although previous investigators had found no false positive results with the PCR method, when DNA contamination was carefully excluded, we did encounter a case of false positive PCR involving a colonic adenocarcinoma.¹⁶ We believe that the apparent monoclonal pattern observed in this case may be related to a combination of a paucity of lymphoid cells in the tissue sample and the presence within this small number of lymphocytes of a clonal reactive population. Nevertheless, the PCR method should still be regarded as being highly specific and the rare cases of false positivity can be easily identified when the results of histology, immunohistochemistry, and immunophenotyping are considered together in the interpretation of the PCR results.

In conclusion, the PCR technique offers major advantages over the conventional Southern blot analysis in terms of its simplicity and rapid turnaround time. It is also the method of choice for the assessment of degraded or size limited samples. The major disadvantage of this method is the low sensitivity of lymphoma case detection which is only 56% in the present study. Nevertheless, the use of this simple and relatively inexpensive method can significantly reduce the number of Southern blot analyses which can be reserved for cases in which the PCR is negative. Future efforts should be directed towards optimising primer design which may subsequently permit detection of a larger proportion of all types of B cell lymphoproliferative disorders. Two recently published

methods are promising in this regard.^{17,18} By using several IgH V region family specific primers corresponding to the first,¹⁷ (Fr1) and second¹⁸ (Fr2) framework regions instead of a single V region primer for the third framework region (Fr3), these authors have documented monoclonality in 94%¹⁷ and 82%¹⁸ of B cell malignancies, respectively. One of these strategies (that using Fr1 primers) is, however, technically more complicated and necessitates the use of a cocktail of seven different primers to achieve the level of sensitivity reported. Therefore, more experience with the use of various primer combinations in different types of B cell proliferations is needed before any one strategy can be regarded as optimal for routine diagnostic purposes.

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