Sources of DNA for detecting B cell monoclonality using PCR

T C Diss, L Pan, H Peng, A C Wotherspoon, P G Isaacson

**Abstract**

**Aims**—To evaluate the polymerase chain reaction (PCR) demonstration of clonal immunoglobulin heavy chain gene rearrangements using routinely prepared, unstained, and stained formalin fixed, paraffin wax embedded tissue samples.

**Methods**—Extracts from (a) fresh frozen tissue samples, (b) unstained, and (c) haematoxylin and eosin stained formalin fixed, paraffin wax embedded 5 μm tissue sections from 42 cases of low grade B cell lymphoma, all shown to be monoclonal by Southern blot analysis, were analysed using PCR. Two regions of the variable segment of the immunoglobulin heavy chain gene were amplified (framework 2 to joining region [Fr2/JH] and framework 3 to joining region [Fr3/JH]). Twelve samples of reactive lymphoid tissue were studied as controls. Products from each case were directly compared on polyacrylamide gels.

**Results**—Using both primer combinations, monoclonality was detected in 38 of 42 (90%) cases using fresh material, 37 of 42 (88%) using unstained paraffin wax embedded samples, and in 35 of 42 (83%) cases using haematoxylin and eosin stained sections. No false positive results attributable to fixation, processing, or staining were identified, although the efficiency of amplification using the Fr2/JH primers was significantly reduced.

**Conclusions**—PCR determination of B cell clonality using paraffin wax embedded material is sufficiently sensitive and reliable for use as a routine diagnostic adjunct to conventional morphological and immunocytochemical assessment of lymphoproliferative disease.

Demonstration of monoclonality at the gene level in B cell lymphomas is of great importance in distinguishing low grade lymphomas from reactive lymphoid lesions, where the morphological and immunophenotypic features may be difficult to interpret. Southern blot analysis of immunoglobulin genes is the established method for molecular genetic demonstration of monoclonality but is slow, complex, and requires fresh frozen tissue samples. It has been proposed that use of the polymerase chain reaction (PCR) can overcome these limitations and offer the rapid and simple detection of B cell monoclonality. The PCR approach is based on amplification of the variable region of the rearranged immunoglobulin heavy chain gene in lymphoid tissue samples, followed by size analysis of products on an electrophoretic gel. Monoclonal B cells give rise to one or two dominant PCR products; polyclonal B cells produce a range of product sizes which appear as a broad smear or ladder on the gel. Most techniques use consensus primers directed to one of the framework regions of the V segment and to the joining region.

These techniques have been evaluated by ourselves and others on a range of lymphoproliferative disorders and have been shown to be highly sensitive and specific. Most work has concentrated on amplification of DNA extracted from fresh tissue samples, from which high purity and high molecular weight DNA can readily be extracted. However, for diagnostic work in the histopathology laboratory, a protocol which works efficiently on fixed, paraffin wax processed tissue is required. This is more demanding as fixation and processing can cause degradation and other changes to DNA. Successful amplification of the immunoglobulin heavy chain gene on material extracted from paraffin wax embedded samples has been reported, but further studies are required to confirm the reliability of the technique as a diagnostic procedure. We have assessed PCR amplification of immunoglobulin heavy chain genes using both unstained and, as paraffin wax blocks are not always available, haematoxylin and eosin stained sections of formalin fixed, paraffin wax embedded tissue from low grade B cell lymphomas.

**Methods**

All material was obtained from the archives of the Department of Histopathology of University College London Medical School, London. Snap frozen, unbuffered formaldehyde fixed, routinely processed paraffin blocks and haematoxylin and eosin stained and mounted sections from 42 cases of low grade B cell lymphoma (five B-lineage chronic lymphocytic leukaemia (B-CLL), 10 centroblastic/centrocytic (CB/CC), two lymphoplasmacytic (LP), 22 mucosa associated lymphoid tissue type (MALT), two mantle

*J Clin Pathol 1994;47:493–496*
cell^9 (MC) and one marginal zone cell^10 (MZC), all showing clonal rearrangement of immunoglobulin heavy chain gene by Southern blot analysis, were analysed. The snap frozen, unfixed tissue samples had been analysed by PCR in a previously published study. Twelve paraffin wax embedded and haematoxylin and eosin stained reactive lymphoid tissue samples (nine tonsils, three lymph nodes) were used as controls.

PREPARATION OF DNA
High molecular weight DNA was prepared from snap frozen, unfixed tissue samples using standard phenol-chloroform extraction. Extracts from unformalised fixed, paraffin wax embedded samples were made using adaptation of a published method. A single 5 μm section of each case was cut and placed into a 0.5 ml microtube using a clean toothpick. The blade was cleaned meticulously between blocks using xylene and a new toothpick was used for each section. Samples were dewaxed using xylene, treated with absolute ethanol as described, and dried before adding 100–200 μl of digestion buffer (10 mM TRIS (pH 8.3), 50 mM KCl, 200 μg/ml proteinase k (Sigma UK), 0.1% Triton X-100). After overnight incubation at 37°C the sections were heated at 95°C for 10 minutes to destroy protease activity, spun at full speed on a microcentrifuge for two minutes, and 2 μl, 5 μl, and 10 μl of the supernatant fluid used in the PCRs.

Haematoxylin and eosin stained paraffin wax embedded sections were obtained from the departmental archives, or freshly stained from the archival blocks using Harris’s haematoxylin and a standard protocol. The coverslips and mountant were removed by soaking in xylene and the sections taken through alcohols to water. After rinsing in distilled water to remove most of the eosin the sections were scraped from the slides using a new scalpel blade and placed into 0.5 ml microtubes before adding digestion buffer, incubation, and heat treatment as described above. Aliquots of 2 μl, 5 μl, and 10 μl were used in the reactions. Extractions from frozen tissue, paraffin wax sections, and haematoxylin and eosin stained sections were made at different times to minimise cross-contamination.

POLYMERASE CHAIN REACTION
PCR amplification of both the Fr3 to joining region (Fr3/JH) and Fr2 to joining region (Fr2/JH) of the immunoglobulin heavy chain gene were performed on all samples using the semi-nested procedures described before. In each case a first round of 30 cycles of amplification of the test DNA extracts with primers Fr3 (ACACGCC[CT]/GC/TGT-ATT ACTGT) or Fr2 (TGG[A/G]TCGG-[C/A]CAG[GC][CT/CC]TT/C/CNGG) with LJH (TGAGGAGACGGTGACC) was followed by 20 cycles of amplification with an aliquot of 0.5 μl from the first round, using primers Fr3 or Fr2 with VLJH (GTGACCA-GGTTNCCTTGCCCCAG). Each reaction contained 10 mM TRIS (pH 8.3), 50 mM KCl, 200 μM each dNTP, 250 ng each primer, 0.1% Triton X-100, and 0.5 units of Taq polymerase (Promega UK). For Fr2/JH amplification, a magnesium chloride concentration of 4.5 mM was used in the first round and 2 M used in the second. For Fr3/JH the concentrations were 9 mM and 3.5 mM. Products were electrophoresed for one hour at 150 volts on 5% (Fr2) or 10% (Fr3) polyacrylamide gels, stained in ethidium bromide, and viewed under ultraviolet light. PCR products from the three preparations were run side by side on the gels for each lymphoma case to confirm that the same sized fragments were amplified.

PCR products for analysis were separated from the amplification and reagent preparations to reduce the risk of contamination. The lymphoma samples and reactive lymphoid controls were amplified on at least two separate occasions, and, in each experiment, were accompanied by a monoclonal sample (Raji cell line or previously characterised B cell tumour), a polyclonal sample (reactive tonsil or lymph node), a negative control with no template DNA, and a further negative control containing the extraction reagents with no tissue sample.

Results
Following amplification with Fr3/JH primers, a monoclonal pattern of one or two discrete bands within the expected size range (65–120 base pairs) was seen in 34 of 42 (81%) cases of lymphoma using both fresh frozen and unstained paraffin wax embedded tissue extracts. The remaining eight cases gave rise to a smear of products. Using haematoxylin and eosin stained sections, 32 of 42 (76%) extracts produced a monoclonal pattern, eight cases produced a smear (the same cases as above), and two yielded no products (tables 1 and 2; fig 1). All reactive controls gave rise to a smear.

After Fr2/JH amplification of fresh tissue
Use of PCR for archival lymphoma tissue

Figure 1 Polyacrylamide gels showing Fr3/JH PCR products. Lane M = Phi X/HindIII markers; (the 100 base pair fragment is indicated on the left); N = negative control (no DNA); C = monoclonal control (Raji cell line); T = polyclonal control (tonsil DNA). F = fresh frozen tissue extract; P = unstained paraffin wax embedded tissue extract; H = haematoxylin and eosin stained tissue extract for lymphoma cases as follows: 1 = MALT lymphomas; 2, 4, and 5 = MALT lymphomas; 3 and 7 = B-CLL; 6 = mantle cell lymphoma. Samples 3 and 5 show two bands, representing amplifiable rearrangement of both alleles.

extracts 36 of 42 (86%) cases showed a monoclonal pattern of one or two discrete bands between 240 and 280 base pairs, while six produced a smear. With unstained paraffin wax embedded samples, 28 of 42 (67%) were monoclonal, two produced a smear, and 12 were negative (no amplification). Using haematoxylin and eosin stained material, 15 of 42 (36%) were monoclonal, one case showed a smear, 24 were negative, and two showed a weak pattern of several bands which varied on repeat amplification, suggesting an inadequate extract (tables 2 and 3; fig 2). Apart from these two cases, all discrete bands seen from a given lymphoma case with each primer set were of the same size (figs 1 and 2), and were reproducible on repeat amplification. Eleven of 12 reactive lymphoid tissue samples showed a polyclonal pattern of amplification from unstained paraffin wax embedded tissue, and 10 of 12 haematoxylin and eosin stained sections also produced a smear. The remainder were negative.

Only a small reduction in yield of PCR product was seen following Fr3/JH amplification from paraffin wax embedded and stained samples when compared with the fresh frozen extracts, but the intensity of bands on the gels was often noticeably reduced after Fr2/JH amplification from paraffin wax embedded and stained tissues.

Discussion

By using PCR to detect monoclonality in low grade B cell lymphomas with Fr3/JH primers, we have achieved equivalent results on unstained formalin fixed, paraffin wax embedded sections and fresh frozen material (81% detection). A similar false negative rate of 20–30% has been published in several studies, although a study of mainly follicle centre cell lymphomas showed a negative rate of 44%. Our detection rate was slightly decreased (76%) when amplification was carried out using haematoxylin and eosin stained, formalin fixed, paraffin wax embedded sections. However, with Fr2/JH primers, the rate was significantly reduced from that achieved using fresh/frozen extracts (86%) when amplification from unstained paraffin wax embedded material (67%) and haematoxylin and eosin stained sections (36%) was attempted. Results were improved for all sample types using both primer pairs, to 90% with fresh tissue samples, to 88% with unstained paraffin wax embedded sections, and to 83% with haematoxylin and eosin stained sections. Except for the negative results, no apparent artefacts were introduced as a result of tissue processing or staining. All reproducible discrete bands amplified, using each procedure, were of identical size for a given patient. Thus a positive result using paraffin wax embedded material was reliable, but a negative result in which no PCR products were seen was unhelpful.

The efficiency of PCR amplification using Fr2/JH primers was reduced on paraffin wax embedded and stained material, in contrast to our findings with Fr3/JH. The Fr2/JH target sequence (240–280 base pairs) is at least twice the length of the Fr3/JH product (65–120 base pairs). This may account for the reduction in sensitivity, as DNA is probably degraded by fixation, processing, and staining, although it is also possible that the Fr2/JH primers are generally less efficient and are compromised by an impure template. Even using Fr3/JH primers, we have experienced a PCR failure rate (in which no products were amplified) of about 20% using referred paraffin wax blocks, suggesting that fixation or processing factors can inhibit the reaction, as reported before.

A single 5 μm section of lymphoid tissue of around 0.5 cm² was sufficient for a minimum of 10 analyses, although extraction efficiency was found to vary between samples. Use of a range of volumes of tissue extract was necessary to achieve successful amplification in

Table 3 Fr2/JH PCR results obtained from frozen, paraffin wax embedded, and haematoxylin and eosin stained material for each lymphoma type

<table>
<thead>
<tr>
<th>Sample</th>
<th>No of cases</th>
<th>Frozen</th>
<th>Paraffin wax</th>
<th>Haematoxylin and eosin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>M</td>
<td>P</td>
<td>N</td>
<td>M</td>
</tr>
<tr>
<td>MALT</td>
<td>22</td>
<td>18</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>CB/CC</td>
<td>10</td>
<td>8</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>B-CLL</td>
<td>5</td>
<td>5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>LP</td>
<td>2</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>MZC</td>
<td>2</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>MZC</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>42</td>
<td>36</td>
<td>6</td>
<td>0</td>
</tr>
</tbody>
</table>

M = monoclonal pattern of one or two discrete bands in the appropriate size range. P = polyclonal pattern of a smear of PCR products. N = negative (no products).
Figure 2 Polyacrylamide gels showing Fr2/JH PCR products. Lane M = Phi X (HindII markers (the 249 base pair fragment is indicated on the left); N = negative control (no DNA); C = monoclonal control (previously characterised B cell lymphoma); F = fresh frozen tissue extract; P = unsection paraffin wax embedded tissue extract; H = haematoxylin and eosin stained tissue extract for lymphoma cases as follows: 1, 4, and 6 = MALT lymphomas; 2 and 3 = CBCC lymphomas; 5 = B-CLL.

some cases, as inhibitory chemicals seem to be present in variable amounts in extracts from paraffin wax embedded and stained sections. Repetition of amplification and direct comparison of products permits the recognition of inadequate DNA samples which may amplify rare polyclonal B cells and give a variable false positive result, or apparently oligoclonal result.

The analysis of PCR products on polyacrylamide, rather than agarose, gels is essential to provide sufficient resolution for this comparison and it also enhances detection of dominant bands within background smears. Blocks and stained sections up to 10 years old were successfully amplified in this study; we have also achieved amplification from paraffin wax blocks up to 30 years old, and anticipate that much older material could also be analysed in this way.

At present we favour the use of Fr3/JH primers, but as these sometimes do not amplify tumour sequences an unexpected polyclonal result may be followed by use of Fr2/JH amplification, although the benefit of this approach on haematoxylin and eosin stained material may be marginal due to a high failure rate. Improvements in sample preparation techniques and PCR parameters are currently being sought to overcome this problem.

It has been reported that monoclonality can be demonstrated in about 80% of low grade B cell lymphomas using immunocytochemistry to show light chain restriction. However, this technique can be difficult to carry out and to interpret, and some lymphomas do not express light chains. PCR may be able to overcome these limitations.

The ability to amplify immunoglobulin sequences from stained tissue sections means that specific anatomical areas and cell types can be selected before PCR amplification, thus facilitating the study of aspects of tumour biology, such as onset, dissemination, and recurrence. We have also been able to use sections treated with other stains, such as periodic acid Schiff and immunocytochemical techniques; these slides are therefore a source of further material.

The present study has confirmed that PCR amplification of immunoglobulin heavy chain gene, particularly using Fr3/JH primers, is a reliable method for the demonstration of B cell monoclonality in extracts from formalin fixed, routinely processed, paraffin wax tissue samples. Furthermore, haematoxylin and eosin stained paraffin wax sections are also a suitable source of DNA for assessment of clonality, thus allowing analysis in the absence of paraffin blocks. The technique described is sufficiently simple, reliable, and easy to interpret for its introduction as a routine procedure for clonal evaluation of B cell lymphoproliferative disease.

Supported by grants from the Cancer Research Campaign and the Leukaemia Research Fund.

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doi: 10.1136/jcp.47.6.493

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