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Detection of clonal B cells in microdissected reactive lymphoproliferations: possible diagnostic pitfalls in PCR analysis of immunoglobulin heavy chain gene rearrangement

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Abstract

***Aims*—To evaluate the specificity of standard and fluorescence based (GENESCAN) polymerase chain reaction (PCR) immunoglobulin heavy chain (IgH) gene rearrangement analysis in complete and microdissected paraffin wax embedded sections from lymphoid proliferations.**

***Methods*—PCR IgH gene rearrangement analysis of whole sections and microdissected fragments (n = 62) from paraffin wax embedded reactive lymph nodes (n = 6) and tonsils (n = 3). Amplificant analysis used both standard methods and automated high resolution fluorescence based quantification and size determination using GENESCAN software.**

***Results*—Whole tissue sections were consistently polyclonal in control experiments. IgH gene amplification was successful in 59 of 62 microdissected fragments; only two of 59 showed a polyclonal rearrangement pattern, the remainder being oligoclonal or monoclonal. Reanalysis was possible in 33 samples; six showed reproducible bands on gel analysis and satisfied accepted criteria for monoclonality. Use of high resolution gels with GENESCAN analysis improved sensitivity and band definition; however, three samples still appeared to be monoclonal.**

***Conclusions*—These results confirm that PCR based IgH gene rearrangement analysis is a sensitive and specific method for demonstrating B cell clonality in whole paraffin wax embedded sections. However, oligoclonal and monoclonal rearrangement patterns are regularly encountered in small tissue fragments from otherwise unremarkable reactive lymphoproliferations, possibly because of preferential priming or detection of local B cell clones. Data from clonal analysis of small, microdissected or lymphocyte poor samples must be evaluated critically. It is recommended that analyses should be run in parallel on at least two tissue specimens. Only reproducible bands present in more than one sample should be considered to be suggestive of neoplasia.**

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Keywords: B cell clonality; GENESCAN; paraffin wax embedded section microdissection; polymerase chain reaction; reactive lymphoproliferation

The histopathological diagnosis of early, borderline, or residual B cell malignancy may be difficult using morphology alone. Diagnostic accuracy can be improved by demonstrating B cell monoclonality using either immunohistochemistry or in situ hybridisation to detect immunoglobulin (Ig) light chain restriction. However, these techniques cannot be used in all cases and may be difficult to interpret—for example, if only a minor lymphomatous component is present in a tissue.

B cell clonality can also be studied by analysis of Ig gene rearrangements in extracted DNA. Traditionally, this has been done using Southern blotting[1]; however, this is a laborious technique that requires large amounts of high quality DNA, which cannot be obtained from fixed, paraffin wax embedded tissue blocks. Recently, various studies have reported the use of rapid, simple methods for the detection of B cell monoclonality using the polymerase chain reaction (PCR) to amplify the variable region of rearranged Ig heavy chain (IgH) genes in lymphoid cells.[2–10] The configuration of an IgH gene rearrangement is unique to an individual B cell and its progeny and can, therefore, be used as a clonal marker. The PCR methods described have used a variety of consensus primers that hybridise with conserved sequences present in most of the variable and joining regions of the IgH gene (VH and JH, respectively). The size of amplified products is usually analysed by gel electrophoresis, with monoclonal populations of B cells giving rise to one or two dominant bands, while polyconal lesions give a range of product sizes, which appear as a smear or ladder of bands in the gel. PCR based techniques are not only simpler, safer, and more rapid compared with Southern blotting, but can also be used to amplify small amounts of poorer quality DNA, such as can be extracted from formalin fixed, paraffin wax embedded blocks, allowing Ig rearrangements to be detected in routinely prepared material.[5 6 11–13] As a result, assessment of B cell clonality by PCR is rapidly gaining acceptance for use in the routine differential diagnosis of malignant lymphoma and reactive lesions.

Many previous studies have concentrated on the sensitivity of PCR methods for detecting IgH clonality. In general, the techniques have been thought to be highly specific, with only occasional reports of false positive monoclonality in reactive lymphoid or non-lymphoid lesions.[14–16] However, the use of PCR Ig gene analysis is being increasingly advocated for

analysing clonality in small tissue biopsies,[17–19] in fine needle aspiration specimens,[15] and even in small groups of cells microdissected from tissue sections,[19–21] or scraped from glass slides of cytological material.[22] The limits of specificity in these circumstances are much less clear. Indeed, some PCR studies using small or microdissected specimens have reported relatively high rates of B cell monoclonality in apparently reactive conditions, often involving extranodal sites such as skin, stomach, salivary gland, or orbit.[18 23–28] This suggests that "false positive" results may be more common than previously realised when using these techniques to define clonality in routine diagnosis.

We examined this problem by using PCR to analyse IgH rearrangements in complete and microdissected paraffin wax embedded sections from reactive lymph nodes and tonsils. We also looked at whether the use of a fluorescence based PCR technique with automated high resolution analysis of amplificants (GENESCAN) could improve diagnostic precision.

Materials and methods

Standard[29] and in house procedures were used, with great care to avoid contamination as a possible source of false positivity during the experiments.

TISSUES

Paraffin wax blocks from six lymph nodes and three tonsils showing reactive hyperplasia and from four patients with nodal B cell non-Hodgkin's lymphoma of various types were obtained from the archives of the Institute of Pathology, Aarhus University Hospital. All tissues were surgically removed for routine diagnostic purposes and had been fixed in buffered formalin and embedded in paraffin wax before being stored routinely.

MICRODISSECTION

Sets of three serial 5 μm sections were cut on to individual glass slides. Slides were stained with haematoxylin and eosin. One section from each set was used as a guide for identifying areas of interest; the second section was microdissected; the third section was scraped in toto for whole section PCR. The guide section was coverslipped; the other sections were dehydrated in graded alcohol and air dried.

Using the guide section for comparison, an area of interest (for example, a germinal centre or interfollicular region) was identified under the ×4 objective. This could be facilitated by lowering the condenser lens when necessary. A 2 μl drop of distilled water was placed over the area using a Pasteur pipette with a new tip with an oblique head. A fine gauge needle was used to microdissect the area manually. First, the needle was used to cut around the region to be removed and this was then scraped loose until the tissue fragment floated in the water drop. Finally, the water was aspirated together with the tissue into the Pasteur pipette and this was transferred into a 1.5 ml Eppendorf tube containing 20 μl 0.28 mM proteinase K in digestion buffer (50 mM Tris, 1 mM EDTA, 0.5% Tween-20, pH 8.5). In some studies, the same germinal centre was identified and sampled in several adjacent sections to assess clonal variation within individual germinal centres. In each case, at least one entire section was scraped completely into a single water drop using a fresh sterile scalpel blade. The tissue and water were then transferred into a 1.5 ml Eppendorf tube containing 200 μl 0.28 mM proteinase K in buffer. To identify possible contamination occurring during manipulation of the section before microdissection, the water drop from some sections was collected without scraping, placed in an Eppendorf tube containing 20 μl 0.28 mM proteinase K in buffer, and processed in the same way as the other samples. These sections were then sampled using a second water drop as described above.

DNA EXTRACTION

The whole and microdissected sections were incubated in 200 μl and 20 μl digestion buffer, respectively, containing 0.28 mg/ml proteinase K, at 55°C for 48 hours in a heating block (Driblock DB-30; Techne, Cambridge, UK). Proteinase K activity was inactivated by heating to 95°C for 20 minutes. The supernatant was used as the template DNA for PCR amplification.

PCR PROCEDURE

Semi-nested PCR for amplification of the junction between V region framework (Fr) III and the joining region of the rearranged IgH gene was performed with primers FR3A (ACA CGG C[C/T][G/C] TGT ATT ACT GT) and LJH (TGA GGA GAC GGT GACC) in the first round and with FR3A and VLJH (GTG ACC AGG GTN CCT TGG CCC CAG) in the second round.[11] The reaction mixture for first round PCR contained 0.2 mM of each dNTP, 10 μl template DNA, 400 ng of each primer, 1 U Taq polymerase (Perkin Elmer, Foster City, California, USA), 2 mM $MgCl_2$, 5 μl of 10× PCR buffer II (Perkin Elmer), and distilled water to a total volume of 50 μl. The PCR programme consisted of 30 cycles of 96°C for 15 seconds, 63°C for 30 seconds, 72°C for 30 seconds, and a final extension at 72°C for 10 minutes. The reaction mixture for the second round PCR contained 0.1 mM of each dNTP, 200 ng of each primer, 1 U Taq polymerase, 3 mM $MgCl_2$, 5 μl of 10× PCR buffer II, and 1 μl PCR product from the first round as template DNA. Distilled water was added to a total volume of 50 μl. The PCR programme comprised 25 cycles with the same time and temperature conditions as for the first round. Each amplification was repeated to study reproducibility.

PCR products were electrophoresed in Visigel separation matrix (Stratagene, La Jolla, California, USA) and visualised using ethidium bromide. In selected experiments, PCR products were also analysed by automated high resolution fluorescence quantification and size determination. In these cases, the second round VLJH primer was fluorescently labelled and 1 μl of the PCR product was mixed with 3 μl formamide and 0.5 μl GENESCAN-2500™ ROX internal size standard (Applied Biosys-

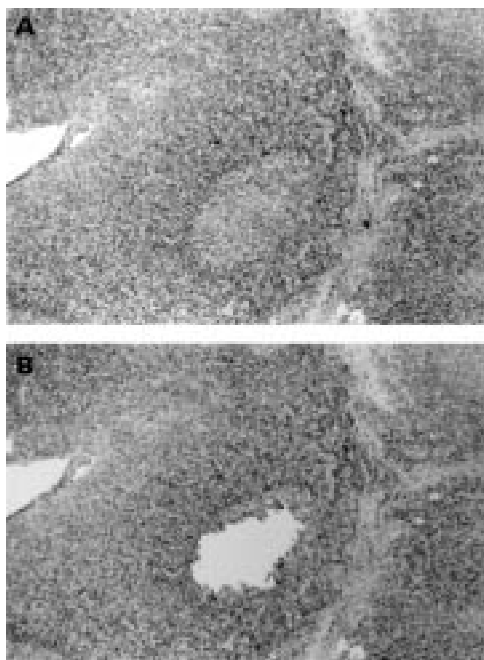

Figure 1 Paraffin wax embedded section from a reactive tonsil (patient 7), before (A) and after (B) microdissection of part of a germinal centre (haematoxylin and eosin stained).

tems, Foster City, California, USA). After denaturation for two minutes at 90°C, the products were separated on a high resolution 6% polyacrylamide gel and analysed using an ABI 373A automated DNA sequencer with GENESCAN 672 software (Applied Biosystems).

CONTROLS
Four cases of nodal B cell non-Hodgkin's lymphoma were used as positive monoclonal tissue controls. In addition, paraffin wax embedded cell pellets from an Epstein-Barr virus infected B cell line (Daudi) and a reactive tonsil served as monoclonal and polyclonal controls, respectively. The PCR reaction mixture without template DNA was used as a negative control. Contamination controls were as described above.

Results

The manual microdissection technique described required some practice to perfect, but could then be performed with relative ease and reproducibilty to remove small tissue areas—for example, complete or partial germinal centres (fig 1). The average diameter of samples was 310 μm for germinal centres and 380 μm for interfollicular specimens. However, samples as small as 100 μm in diameter could be accurately removed if required. Sampling of smaller groups of cells is more difficult and requires a semi-mechanical technique (not shown).

CONTROLS
Positive controls for Ig heavy chain rearrangement gave appropriate results in all cases (figs 2 and 3). PCR analysis of whole sections from the monoclonal B cell non-Hodgkin's lymphoma controls gave one or two distinct bands in the expected range (~ 70–120 bp).

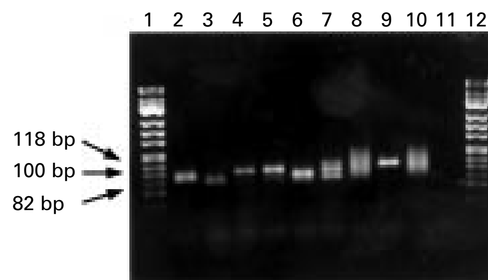

Figure 2 Gel electrophoresis of FRIII/JH PCR products. Lanes 1 and 12, ØX/HinfI molecular weight marker; lanes 2 and 3, repeat analyses on microdissected germinal centre (patient 3, interfollicular sample 1) with non-reproducible monoclonal bands; lanes 4 and 5, repeat analyses on microdissected germinal centre (patient 5, germinal centre sample 3) with reproducible monoclonal bands; lanes 6 and 7, repeat analyses on microdissected germinal centre (patient 5, germinal centre sample 2), initially monoclonal, subsequently oligoclonal pattern; lane 8, whole section from lymph node (patient 2) showing polyclonal smear; lane 9, B cell chronic lymphocytic leukaemia positive monoclonal control; lane 10, tonsil polyclonal control; lane 11, negative control.

The polyclonal controls showed a smear without discrete bands on gel analysis, and a series of normally distributed peaks of varying height in the GENESCAN electrofluorograms, each major peak differing in size by three base pairs. Using GENESCAN, some peaks showed a shoulder or second top separated by 1 bp from the main peak (fig 3). This is an artefact caused by the tendency of the Taq polymerase that we used to add one random base (usually A) to the 3' end of the strand before it drops off. This artefact can be prevented by using other DNA polymerases (data not shown). In dilution experiments, a monoclonal population of B cells comprising 1–5% in a polyclonal background could be detected in stained gels (data not shown). GENESCAN analysis was more sensitive by approximately one log. The use of electrofluorograms made it much easier to assess PCR products than conventional visual examination of a gel and greatly improved the resolution of bands of similar sizes. No amplified products were obtained from the negative or contamination controls.

In separate dilution experiments performed on whole section polyclonal controls, extracted DNA was diluted sequentially with distilled water before the PCR was performed. These analyses (data not shown in full) revealed the expected initial polyclonal picture, changing to a monoclonal pattern at a dilution factor of 1/10 000.

PATIENTS' SPECIMENS
The results are summarised in table 1 and representative examples are shown in figs 2 and 3. PCR amplification was successful in the six lymph nodes and three tonsils when whole paraffin wax embedded sections were used as the DNA source. In all nine cases, a polyclonal pattern consisting of a smear of amplified products was seen (fig 2). Case 3 was a reactive lymph node in which primary follicles only were present, without well defined germinal centres. Whole section PCR in this case showed initially an oligoclonal pattern (defined as more than two discrete bands) superim-

posed on a polyclonal background. Repeat analysis of an adjacent section from this case showed a polyclonal pattern.

A total of 62 samples were microdissected from the patients' tissues; 41 were from germinal centres and 21 were from interfollicular areas. Amplification was successful in 59 of 62 samples. In patients 6 and 9, one in four and two in six microdissected samples, respectively, could not be amplified. In contrast to the whole section results, only two of the 59 microdissected samples showed a polyclonal pattern. These samples both came from patient 6. In a third sample from patient 6, a monoclonal band on a polyclonal background was found. On initial testing, an oligoclonal or monoclonal pattern was found in 29 and 28 of the 59 informative samples, respectively. In seven samples, the pattern switched from oligoclonal to monoclonal, or vice versa, when analysis was repeated (see patients 1, 2, 4, 5, 7, and 9). Of the 30 samples that were monoclonal on at least one occasion (defined as one or two dominant discrete bands), sufficient material was available for reanalysis in 21; in 14 of these, monoclonality was found in both analyses. Although band size varied in most of these repeat specimens, six of the 14 samples appeared to maintain the same sized band in gel analysis (fig 2). Two of these six specimens were interfollicular samples. However, GENESCAN analysis (fig 3) of the six apparently monoclonal specimens revealed an oligoclonal pattern in three cases. Thus, of the 33 samples analysed twice, six would have been regarded as monoclonal using accepted criteria for

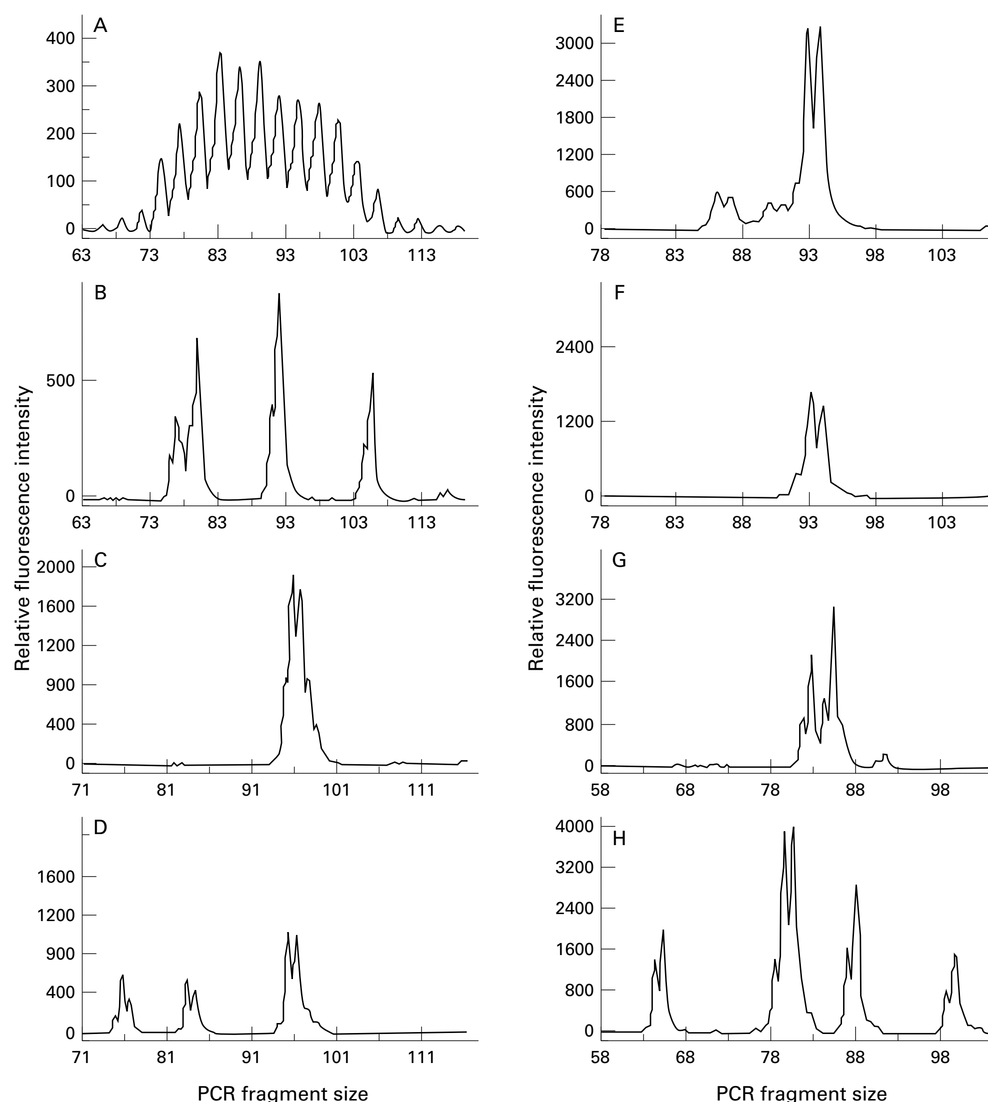

Figure 3 Representative electrofluorograms from GENESCAN analysis of fluorescent FRIII/JH PCR products. Relative fluorescence intensity (y axis) is plotted against PCR fragment size (x axis). Some peaks show a shoulder or second top separated from the main peak by 1 bp. This is an artefact caused by the tendency for Taq polymerase to add a random base to the 3' end of the amplified strand before dropping off (see text). (A) Whole section from reactive tonsil (patient 7) showing polyclonal distribution of peaks. (B–H) Microdissected samples. (B) Lymph node from patient 5 (germinal centre sample 2) showing oligoclonal pattern with several discrete peaks. (C and D) Repeat analyses of lymph node from patient 4 (germinal centre sample 4) showing reproducible dominant band (96 bp). Two additional minor bands are present in the second analysis. (E and F) Repeat analyses of lymph node from patient 3 (interfollicular sample 3) showing reproducible monoclonal pattern with identically sized main peaks (94 bp). (G and H) Repeat analyses of lymph node from patient 1 (germinal centre sample). On agarose gel analysis, these samples showed a reproducible monoclonal pattern with apparently identical single main bands. GENESCAN analysis reveals a switch from monoclonal to oligoclonal patterns. The main peaks are of similar but clearly different sizes.

Table 1 Clonal analysis of paraffin wax embedded reactive lymph nodes and tonsils

Patient	Tissue	Whole section	Microdissected samples: Germinal centres 1	2	3	4	5	6	Interfollicular regions 1	2	3	4	5	6
1	LN	p/p	o/m	o/o	m#/m#	m/o								
2	LN	p/p	o	o	o	m/m	o/o	o/o	o	o	o	m#/m#	o/o	m/o
3	LN	o/p+p	o	o	o				m/m	m/m	m*/m*			
4	LN	p/p	m	m	o	m*/m*	o/o	m/m	o/m	o/o	o/o			
5	LN	p/p	m/m m†	m/o o†	m*/m* o†	m	m#/m#		m/m	o/o	o/o			
6	LN	p/p	nb	p+m	p	p								
7	Tonsil	p/p	o	o	o	m			m/o	o/o	o/o			
8	Tonsil	p/p	m	m	m									
9	Tonsil	p/p	o/o	m/o	m/m				m/m	nb	nb			

Immunoglobulin heavy chain gene rearrangement detected by FRIII-PCR with subsequent gel analysis. Selected experiments were repeated using GENESCAN analysis. Results of experiments repeated on the same sample are separated by an oblique stroke.
#Identical band size on repeat analysis in gel, but not in Genescan.
*Identical band size on repeat analysis in both gel and GENESCAN.
†Same GC analysed from an adjacent section.
LN, lymph node; m, monoclonal; nb, no band; o, oligoclonal; p, polyclonal.

evaluation of Ig gene rearrangement patterns in gels, and three (patient 3 (interfollicular region sample 3); patient 4 (germinal centre sample 4); and patient 5 (germinal centre sample 3)) still appeared to be monoclonal by GENESCAN (table 1). In all cases in which more than one sample was found to be monoclonal, the size of the monoclonal bands differed from area to area (for example, patient 5).

In patient 5, it was possible to sample some germinal centres in two adjacent sections (table 1). In germinal centre sample 1 from patient 5, both samples were apparently monoclonal, but with different (non-reproducible) bands. In germinal centre sample 3 from patient 5, one sample showed a reproducible monoclonal band whereas the same germinal centre sampled from the adjacent section was oligoclonal.

Discussion

PCR analysis of IgH gene rearrangement is a fast, simple, and economical technique that is rapidly becoming the method of choice for the routine diagnosis of B cell clonality. Our study confirms previous reports that the degraded DNA extracted from formalin fixed, paraffin wax embedded tisses can be amplified successfully in most cases, permitting the analysis of clonality in archival material.[5 6 11–13] Amplification of DNA from single, complete, paraffin wax embedded sections was successful in each of the six polyclonal and monoclonal controls we used in numerous experiments, and in each of the nine test lymph nodes and tonsils. The amount of DNA available in the starting material did not appear to be a limiting factor. Even when using small microdissected paraffin wax section fragments, successful amplification was possible in 59 of 62 specimens. The microdissection technique that we used is technically simple and, with practice, easy to perform. False positivity as a result of contamination was not a problem in our study.

In keeping with many previous studies, we were able to detect a monoclonal population of B cells, when these comprised 1–5% against a polyclonal background, using traditional gel electrophoresis. Sensitivity was considerably improved by using fluorescence labelled primers with product separation on high resolution gels and automated GENESCAN analysis. Evaluation of the electrofluorograms was simple, quick, and accurate compared with conventional visual assessment of a gel. Separate bands could be resolved to within a single base pair, facilitating the distinction of clonal populations from background and improving precision in distinguishing polyclonal, oligoclonal, and monoclonal patterns. Other groups have reported comparable results using similar techniques for analysis of IgH and T cell receptor gene rearrangements.[18 30–32]

Previous studies using PCR analysis of B cell monoclonality in lymphatic tissues have reported very few false positive results in reactive conditions, particularly if only reproducible bands were accepted as clonal. This was also true in our study when we used intact sections as the starting material. However, we found a much more complex picture when we examined the microdissected fragments from reactive lymph nodes and tonsils. Of the 59 informative microdissected samples, only two were polyclonal on initial analysis, whereas 30 tested monoclonal at least once. Although most of these bands were not reproducible, six of 33 fully analysed microdissected samples gave apparently identical clonal bands on gel reanalysis. The high resolution possible with GENESCAN analysis proved useful in showing that in three of these cases the repeat bands were actually of different sizes. However, reproducible bands were confirmed in the remaining three cases which would, therefore, have satisfied accepted criteria for monoclonality.

There are a number of possible explanations for our finding of false positive oligoclonality and monoclonality. Several studies have described apparent clonal PCR patterns appearing if analysis is performed on too small a number of reactive lymphocytes, although in most reports the bands were not reproducible.[15 21] A similar mechanism was suggested as an explanation for the false positive IgH gene rearrangement reported by Ling *et al* in a case of colonic adenocarcinoma.[14] Circumstantial evidence that the number of target cells in a test sample may be important comes from our dilution experiments in which whole section tonsil controls

switched from a polyclonal to a monoclonal pattern on serial dilution to 1/10 000. Similar experiments have been described by Wan *et al*.[15] One possible mechanism underlying this phenomenon is preferential priming of VDJ sequences.[15 33] The primer sets used in IgH chain rearrangement analysis will have varying affinity for some Ig gene rearrangements. If the initial number of target sequences is very low, this preferential amplification of certain VDJ configurations may result in a spurious oligoclonal or monoclonal banding pattern which should, however, be non-reproducible. It is unlikely that a paucity of target cells can fully explain our findings because the microdissected specimens we used still contained numerous lymphocytes. An alternative or additional explanation is that the fragments might include local clusters of clonal or clonally related cells, which give a dominant band on amplification. This would also account for those examples in which the monoclonal bands were reproducible. There is good evidence, in part from single cell microdissection analysis,[34] that selection of high affinity B cell clones in antigen stimulated germinal centres results in a gradual evolution from an initially polyclonal germinal centre to one that is dominated by a few large clonally related B cell groups. This phenomenon is particularly evident in the dark zone. Thus, the chances that a microdissected germinal centre will contain a dominant B cell clone would appear to be relatively high. Similarly, small interfollicular tissue fragments may contain clonal clusters generated in response to a specific local antigenic stimulation.

We initiated our study because we were concerned that the widely accepted rules for determining monoclonality in paraffin wax embedded sections of lymphomas using PCR IgH gene analysis might not be sufficient to avoid false positive results when applied to small or microdissected biopsies. Our results confirm that this is true. The frequency with which we could demonstrate apparent oligoclonality or monoclonality in microdissected reactive lymphoid tissues emphasises the care that must be taken in interpretation. Strict adherence to the rule that genuine clonal bands must be reproducible reduces some of the danger of arriving at a spurious diagnosis. In this context, it is reassuring that the use of a highly sensitive technique for product analysis (GENESCAN) decreased rather than increased the risk of error. However, it is still disconcerting that a reproducible monoclonal band pattern could be demostrated in some 10% of our fully analysed reactive tissue fragments. Clearly, the often stated principle that monoclonality does not necessarily denote neoplasia should be emphasised. We examined separate microdissected fragments from each of our patients, from both the same and adjacent sections. Importantly, in none of these did we detect the same band patterns, even when reproducible monoclonality could be demonstrated within an individual fragment. We recommend, therefore, that when investigating possible cases of B cell neoplasia using microdissection, PCR reactions should be run in parallel on at least two separate tissue fragments. Only reproducible bands present in more than one fragment should be considered to be suggestive of neoplasia. Finally, it should also be emphasised that demonstrating identical migration of amplified fragments in a gel does not provide absolute proof of sequence identity (and thus clonality), because the possible size range of products from IgH gene PCR is relatively limited. In case of doubt, PCR products must either be analysed using a technique where migration through a gel is dependent on the precise nucleotide sequence (such as heteroduplex analysis) or they should be sequenced in full.

The relative ease with which we could detect what appear to be small clonal B cell populations is relevant to other studies. High rates of monoclonality have been described by several groups in acquired mucosa associated lymphoid tissue without histological evidence of overt lymphoma.[18 23–27 35] These findings have been variously interpretated to indicate preferential proliferation of benign B cell monoclones,[27] premalignant B cell proliferation,[23] and incipient lymphoma.[35] Using a similar strategy to ours for IgH VDJ amplification with GENESCAN analysis, Calvert *et al* recently reported clonal analysis of microdissected chronic gastritis specimens.[18] They found reproducible monoclonal bands in three of 28 biopsies with apparently benign *Helicobacter pylori* gastritis, and in two of eight specimens with gastritis in association with adenocarcinoma. Similarly, Ritter *et al* used PCR to analyse B cell clonality in skin lymphoproliferations and found possible monoclonality in 18 of 40 benign infiltrates, including four cases that gave reproducible bands.[28] Most of these false positive results occurred in biopsies with scanty, B cell poor infiltrates. These reports and the results of our own study emphasise the dangers of overinterpretation of clonality data. The high sensitivity of PCR means that benign oligoclonal and monoclonal B cell populations will sometimes be detected, not only in certain states of disordered immunity or preneoplasia, but also in otherwise unremarkable reactive lymphatic tissues. Our results confirm that the use of high resolution gels (particularly in combination with automated GENESCAN analysis) has an important part to play in resolving ambiguities and possible false monoclonality when analysing PCR products from both small samples and routine whole sections. Caution must still be exercised in the interpretation of PCR clonality data, particularly when examining small, B cell poor, or microdissection specimens. However, an awareness of the importance of the reproducibility of clonal patterns within a lesion, and careful evaluation of PCR genotyping, together with clinical, morphological, and immunophenotypical data will reduce the risk of misdiagnosis.

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