Cytogenetic and molecular analysis of follicular non-Hodgkin's lymphoma

by

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SUMMARY

The incidence of the reciprocal translocation \textit{t}(14;18)(q32;q21) in follicular lymphoma varies from 50\% to 90\% in American series. In the largest series from Europe, the translocation was present in only 21/51 (41\%) of cases. The wide variety in incidence may in part be due to the method of detection used - usually cytogenetics alone or a combination of Southern blotting and polymerase chain amplification (PCR) of DNA extracted from malignant tissue. The shortcomings of each technique suggest that a combination of all three may best determine the true incidence of the translocation.

This study describes and discusses the cytogenetic analysis of lymph nodes from seventy two patients with follicular lymphoma. Forty nine patients also had DNA extracted from diagnostic specimens for molecular analysis using both Southern blotting and the polymerase chain reaction (PCR) to detect \textit{t}(14;18).

The translocation was found in 76\% using a combination of all three methods. The most common other cytogenetic abnormalities included +7, +der(18), +18, +21, +X and +8. Cytogenetics proved the most comprehensive method of detecting \textit{t}(14;18) and Southern blotting was superior to PCR. The numerous additional karyotypic abnormalities present contributed little to the management of patients with follicular lymphoma.
However, molecular methods detected t(14;18) in samples where karyotyping was unsuccessful and the stability of t(14;18) as a marker of disease was confirmed by the finding of molecular evidence of the translocation after analysis of sequential samples from several patients. The increased sensitivity of PCR allowed detection of t(14;18) bearing cells in samples of marrow and peripheral blood stem cell harvests from patients who were thought by conventional criteria to be in clinical remission. As treatment of follicular lymphoma becomes more aggressive, molecular detection of t(14;18) will become more important in monitoring of treatment and detection of early relapse.
ACKNOWLEDGEMENTS

Knowledge is of two kinds. We know a subject ourselves, or we know where we can find information upon it (Samuel Johnson, 1775).

I wish to thank those who provided the information which made this thesis possible. Firstly, I am grateful to Professor HJ Evans and Dr M Steel for permission to carry out this project and for their support during my time at the MRC Human Genetics Unit. For provision of specimens, molecular probes, histological expertise and encouragement, I thank my advisor, Dr A Krajewski. I am especially indebted to Dr Fiona Ross and to Dr Lynn Hendry for their cytogenetic skills and for introducing me to the mysterious world of chromosomes. Dr D Saltman and Dr Elizabeth Thomson provided valuable advice on molecular techniques and Sandy Bruce provided essential photographic assistance.

I am grateful to the haematologists of Lothian, Fife and the Borders, and also the Kay Kendall Leukaemia Fund, for access to patient samples. Lastly, I wish to thank the staff of the Blood Lab. for their patience, good humour and good company which made my years at the MRC extremely enjoyable.
DECLARATION

I declare that the work in this thesis is my own, unless stated otherwise.

Gillian Turner
March, 1992
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<th>Full Form</th>
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<tbody>
<tr>
<td>ABMT</td>
<td>autologous bone marrow transplant</td>
</tr>
<tr>
<td>bcl-2</td>
<td>B-cell lymphoma/leukaemia-2</td>
</tr>
<tr>
<td>CE</td>
<td>conventional electrophoresis</td>
</tr>
<tr>
<td>dATP</td>
<td>deoxyadenosine-5'-triphosphate</td>
</tr>
<tr>
<td>dCTP</td>
<td>deoxycytidine-5'-triphosphate</td>
</tr>
<tr>
<td>dGTP</td>
<td>deoxyguanosine-5'-triphosphate</td>
</tr>
<tr>
<td>dTTP</td>
<td>deoxythymidine-5'-triphosphate</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
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<tr>
<td>EDTA</td>
<td>ethylene diaminetetra-acetic acid</td>
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<tr>
<td>FL</td>
<td>follicular lymphoma</td>
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<tr>
<td>FLC</td>
<td>follicular large cell lymphoma</td>
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<tr>
<td>FMC</td>
<td>follicular mixed small and large cell lymphoma</td>
</tr>
<tr>
<td>FSC</td>
<td>follicular small cell lymphoma</td>
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<tr>
<td>JH</td>
<td>joining region of immunoglobulin heavy chain gene</td>
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<tr>
<td>mbr</td>
<td>major breakpoint region of bcl-2</td>
</tr>
<tr>
<td>mcr</td>
<td>minor cluster region of bcl-2</td>
</tr>
<tr>
<td>NHL</td>
<td>non-Hodgkin's lymphoma</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute</td>
</tr>
<tr>
<td>SCH</td>
<td>peripheral blood stem cell harvest</td>
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<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
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### CYTOGENETIC TERMS

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<td>del</td>
<td>deletion</td>
</tr>
<tr>
<td>der</td>
<td>derivative chromosome</td>
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<tr>
<td>dic</td>
<td>dicentric</td>
</tr>
<tr>
<td>dup</td>
<td>duplication</td>
</tr>
<tr>
<td>i</td>
<td>isochromosome</td>
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<tr>
<td>ins</td>
<td>insertion</td>
</tr>
<tr>
<td>inv</td>
<td>inversion</td>
</tr>
<tr>
<td>NFA</td>
<td>not fully analysable</td>
</tr>
<tr>
<td>p</td>
<td>short arm</td>
</tr>
<tr>
<td>q</td>
<td>long arm</td>
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<tr>
<td>t</td>
<td>translocation</td>
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CHAPTER 1

INTRODUCTION
INTRODUCTION

Cancer is thought to arise by a multistep process from a single cell of origin (Nowell, 1976). The original clone is genetically unstable and tumour progression occurs through acquisition of secondary genetic changes. The discovery of viral oncogenes and their cellular counterparts established a link between chromosomal abnormalities and neoplasia (Bishop, 1985).

The low-grade, follicular non-Hodgkin's lymphomas provide further evidence for the clonal evolution of malignancy (Yunis et al, 1987). These lymphomas arise from the neoplastic proliferation of B-cells derived from lymphoid tissue, and also from their precursor cells in the bone marrow (Bertoli et al, 1988). They are identified histologically by their growth pattern and resemblance to normal B-cell counterparts (Rappaport, 1966; Gerard-Marchant et al, 1974; Lukes et al, 1974) and can also be classified using panels of B-cell differentiation markers (Foon et al, 1986). Their monoclonal origin has been demonstrated by molecular analysis of immunoglobulin gene rearrangements (Arnold et al, 1983; Cleary et al, 1984). More interestingly, cytogenetic studies have identified a multitude of clonal, recurrent and non-random karyotypic abnormalities in lymph nodes from patients with non-Hodgkin's lymphoma (Fukuhara et al, 1979; Yunis et al, 1982; 1987; Bloomfield et al, 1983; Speaks et al, 1987).
The reciprocal translocation t(14;18)(q32;q21) occurs in 55% (Bloomfield et al, 1983) to almost 85% (Yunis et al, 1987) of those with follicular lymphoma. Cloning of the breakpoints of the translocation led to the discovery of the novel oncogene bcl-2 on chromosome 18q21 (Tsujimoto et al, 1984a; Bakhshi et al, 1985). Using chromosome 18 derived probes, conventional electrophoresis (CE) can identify bcl-2 rearrangements compatible with t(14;18) in DNA extracted from lymphomatous tissue (Weiss et al, 1987). Similarly, amplification of specific sequences from such DNA using the polymerase chain reaction (PCR) can detect t(14;18)-bearing cells in tissue found by conventional histologic and immunologic techniques to be free of disease (Lee et al, 1987a). The detection of such minimal disease has had considerable implications in the diagnosis, staging and treatment of follicular lymphoma. The first part of this introduction will discuss conventional approaches to the classification, diagnosis and staging of low grade lymphoma and then the impact of these new techniques will be considered. The final part of the introduction deals with the aims of this thesis.

1.1 Morphology and immunology of follicular lymphoma

The non-Hodgkin's lymphomas (NHL) are a complex and diverse group of neoplasms of which the follicular lymphomas form a distinct morphological and biological subgroup. The
early classifications of NHL were based on the growth pattern and morphological features of the malignant cells (Rappaport, 1966). The more recent classifications of NHL have taken into account both immunological findings and the clinical aggressiveness of the different sub-groups of lymphoma (Gerard-Marchant et al, 1974; Lukes and Collins, 1974). Attempts to relieve the confusion produced by the multiple classifications resulted in the Working Formulation (Rosenberg, 1982), which is used throughout this thesis. This separates NHL into ten major types using morphological criteria only and permits translation among the other systems and comparisons of clinical trials. Table 1.1 gives the Working Formulation and the equivalent Kiel classification (Gerard-Marchant et al, 1974) of the follicular non-Hodgkin's lymphomas. Groups B, C and D in the Working Formulation correspond to the Rappaport designations of nodular, poorly differentiated lymphocytic; nodular, mixed lymphocytic and histiocytic and nodular histiocytic lymphoma respectively (Rappaport, 1966).

Follicular lymphomas are comprised of small cleaved cells (centrocytes) and large and small non-cleaved cells (centroblasts) which resemble the transforming B-cells in reactive lymphoid follicles (Nathwani et al, 1981)(Figure 1.1). The neoplastic follicles may be composed predominantly of centrocytes or centroblasts or a mixture of both. The
Table 1.1  Working Formulation of follicular non-Hodgkin's lymphomas for clinical usage, with equivalent Kiel classification

<table>
<thead>
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<th>Working Formulation</th>
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<tr>
<td><strong>Low grade</strong></td>
<td></td>
</tr>
<tr>
<td>B. Malignant lymphoma, follicular</td>
<td></td>
</tr>
<tr>
<td>Predominantly small cleaved cell</td>
<td></td>
</tr>
<tr>
<td>diffuse areas</td>
<td>} ML centroblastic-centrocytic (small),</td>
</tr>
<tr>
<td>sclerosis</td>
<td>follicular +/- diffuse</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>C. Malignant lymphoma, follicular</td>
<td></td>
</tr>
<tr>
<td>Mixed, small cleaved and large cell</td>
<td></td>
</tr>
<tr>
<td>diffuse areas</td>
<td></td>
</tr>
<tr>
<td>sclerosis</td>
<td></td>
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<td></td>
<td></td>
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<tr>
<td><strong>Intermediate grade</strong></td>
<td></td>
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<tr>
<td>D. Malignant lymphoma, follicular</td>
<td></td>
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<tr>
<td>Predominantly large cell</td>
<td>ML centroblastic-centrocytic (large),</td>
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<tr>
<td>diffuse areas</td>
<td>follicular +/- diffuse</td>
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<td>sclerosis</td>
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Figure 1.1  Follicular non-Hodgkin’s lymphoma. Section of lymph node. Case 10. Haematoxylin and eosin. x 40.
malignant cells may be apparently confined to the follicles or may diffuse into the interfollicular spaces. Occasionally, the follicles may be separated by dense bands of collagen, giving a sclerotic appearance to the node.

In addition to morphologic classification, the development of complex panels of monoclonal antibodies which identify cell surface activation and differentiation antigens has enabled NHL to be specifically immunophenotyped. As well as histologically resembling normal stages of B and T-cell development, the tumour cells express unique immunologic phenotypes which are comparable to stages of normal B or T-cell differentiation (Urba et al, 1985). Follicular lymphoma cells possess surface immunoglobulin and are also positive for many other cell-surface markers, including HLA-DR, CD19, CD20 and CD24 and often CD21. Up to 50% may be CALLA positive (CD10) but, unlike chronic lymphatic leukaemia, cells are negative for CD5. Their immunophenotype reflects that of mature follicle B-cells (Foon et al, 1986; Mulligan, 1990) (Figure 1.2).

Immunophenotyping of NHL has been instrumental in distinguishing B-cell from T-cell lymphomas (Pinkus et al, 1979; Foon et al, 1986). A further useful function is in distinguishing lymphoid from non-lymphoid tumours (Sloane, 1987), particularly in adults where small and large cell carcinomas may be confused with low and high grade...
Figure 1.2 Expression of B-cell cluster designated antigens. Follicular lymphoma cells possess surface immunoglobulin, HLA-DR, CD19 and CD20 and phenotypically resemble the mature B cells of lymph node follicles.
lymphomas. Where the diagnosis is not in doubt however, the additional information provided by immunophenotyping is frequently of neither prognostic nor diagnostic significance. Salter et al (1989) concluded that phenotyping B-cell NHL for many of the antigens expressed during B-cell differentiation and activation did not provide clinically useful information in addition to that obtained from standard histological classification.

The immunocytochemical detection of surface immunoglobulin and the molecular detection of immunoglobulin gene rearrangements are particularly important in B-cell malignancies. The successful rearrangement of immunoglobulin heavy and light chain genes is an essential step in the development of B-cells. The structure of immunoglobulin molecules and the genetic events leading to their synthesis have been extensively reviewed (Waldmann, 1987; Mulligan, 1990). The immunoglobulins are coded for by three gene families on three different chromosomes. The heavy chain (H) genes are located on chromosome 14q32 (Croce et al, 1979), the kappa (k) light chain genes on chromosome 2p11 (Malcolm et al, 1982) and the lambda light chain locus is on chromosome 22q11 (Erikson et al, 1981). Each set of genes consists of discontinuous segments of DNA which rearrange to form a productive immunoglobulin gene. Every immunoglobulin molecule consists of two light and two heavy chains. Each heavy or light chain
contains variable (V) regions which bind antigen and constant (C) regions which determine immunoglobulin class and biological activity. As well as V and C regions, all three loci contain joining (J) segments and, in the case of the heavy chain locus, a set of diversity (D) genes. The rearrangement of the heavy chain VDJ segments occurs at the pre-B cell stage of development and marks the definite commitment of the cell to the B-cell lineage (Korsmeyer et al, 1981) (Figure 1.3). The selection of V, D and J regions is virtually random and antibody diversity at this stage is at the expense of B-cells as the 80% which fail to generate a productive gene simply die. A successful VDJ recombination prevents recombination of the other allele (Alt et al, 1982; 1984). Further diversity is generated by the imprecise breakpoints within the VJ and VDJ segments, with variability leading to amino acid substitution at certain key loci. The insertion of extra nucleotides during recombination (N sequences) increases junctional diversity even more (Alt et al, 1982). Lastly, the high rate of somatic mutation within the variable regions also leads to nucleotide substitution (Tonegawa, 1983).

The next event in B-cell development is rearrangement of the light chain genes, kappa before lambda. A successful kappa rearrangement excludes rearrangement of the lambda light chain genes, and a cell will continue to secrete only one type of light chain (Froland et al, 1972). The secretion
Figure 1.3 Schematic representation of immunoglobulin heavy chain gene rearrangements, leading to a functional VDJ rearrangement and synthesis of cytoplasmic IgM.
of immunoglobulin heavy chains is more complex. Transcription of the heavy chain constant locus leads to synthesis of IgM initially but a mechanism of heavy chain class switching allows synthesis of IgM and IgD concurrently (Kataoka et al, 1980; Teale et al, 1987) and eventual production of IgA, IgE or IgG, but always in association with the same light chain class. The excluded allele may remain in the germline configuration, be rearranged aberrantly or be deleted.

In normal peripheral blood and lymph nodes there is usually a mixture of kappa and lambda light chain secreting B-cells, with two thirds of the population secreting kappa. Because B-cell lymphoid neoplasms usually develop from a single cell of origin, they secrete only one light chain isotype. Therefore, the detection of clonal excess of one light chain type is highly suspicious of malignancy (Figure 1.4). Using sensitive flow cytometric techniques to detect monoclonal light chain secretion, circulating malignant cells can be found in the peripheral blood of patients with lymphoma (Berliner et al, 1986). Occasionally, B-cell lymphomas may fail to secrete immunoglobulin or the clonal population may be too small to be detected by immunohistological methods. The molecular detection of rearrangements of the immunoglobulin genes can overcome these problems (Davis et al, 1991). As mentioned before, the population of B-cells within a lymph node is polyclonal and analysis of immunoglobulin
Figure 1.4  Follicular non-Hodgkin's lymphoma. Case 10. Immunocytochemical detection of kappa light chain. x 250.
gene rearrangements by Southern blot hybridisation shows no specific rearranged band. However, analysis of immunoglobulin genes from B-cell lymphoma nodes reveals the monoclonal origin of these tumours and a unique, identifiable pattern of bands is seen in each tumour (Arnold et al, 1983; Cleary et al, 1984). Similar techniques exploit the T-cell receptor genes in T-cell malignancies (Croce et al, 1985; 1986; Reis et al, 1989; Griessner et al, 1989). The detection of T-cell receptor and immunoglobulin gene rearrangements can be helpful in distinguishing B from T-cell disease (Arnold et al, 1983) but frequently provides little additional information to that obtained from immunophenotyping (Henni et al, 1988). Molecular techniques can detect minor populations of neoplastic lymphocytes comprising 1% of the total cell population (Cleary et al, 1984) and can detect circulating lymphoma cells in peripheral blood before and after treatment (Horning et al, 1990). The presence of circulating malignant cells at diagnosis was commonest in those with low-grade disease, reflecting the increased incidence of marrow involvement and was rarely seen in those with high grade lymphoma. Detection of clonal cells in the peripheral blood after treatment was not predictive of relapse and the significance of such minimal residual disease is not yet known (Horning et al, 1990). Sequence analysis of the productive immunoglobulin heavy chain locus has revealed the monoclonal origin of apparently bigenotypic and biphenotypic follicular
lymphomas where the productive allele had undergone a heavy chain class switch (Cleary et al., 1988).

Many of the disadvantages of conventional electrophoresis such as the time involved and the complexity of the technique can be overcome by the use of the polymerase chain reaction and consensus primers for the V and J regions of the immunoglobulin gene (Trainor et al., 1990). This technique is faster and simpler and at least as sensitive but unfortunately fails to detect monoclonality in all cases of B-cell malignancy.

1.2 Clinical features of follicular lymphoma

The majority of patients with follicular lymphoma present with lymphadenopathy and the diagnosis is usually made by nodal biopsy. The extent of disease at diagnosis is determined by various staging procedures based on those devised for Hodgkin's lymphoma (Carbone et al., 1971). Necessary investigations include computerised tomography of the chest and abdomen, chest X-ray, bone marrow biopsy and routine haematological and biochemical profiles. Bone marrow involvement is present in approximately 40% of patients at diagnosis using morphological criteria (Gallagher et al., 1986) but the incidence may rise to 80% when the more sensitive immunological methods described in Section 1.1 are used (Ault, 1979; Berliner et al., 1986). After staging,
therefore, 10-20% of patients have localised (stage I or stage II) disease, the remainder having more disseminated (stage III or IV) lymphoma.

Both the histologic group and the stage of lymphoma are crucial in predicting prognosis. The median survival of patients with follicular lymphoma is between 4 and 10 years (Horning et al, 1984; Gallagher et al, 1986) which is significantly better than diffuse lymphocytic lymphoma (Anderson et al, 1982). Those with small cell disease tend to do better than those with follicular large cell lymphoma (Rudders et al, 1979). The presence of B symptoms, advanced (Stage IV) or bulk disease at diagnosis are all poor prognostic features (Rudders et al, 1979; Anderson et al, 1982). In those who die of follicular lymphoma, symptoms of bone marrow failure are common and in approximately 50% the disease retains a follicular pattern. In the majority of the remainder, the disease becomes more aggressive and unresponsive to treatment. Rebiopsy of lymph nodes reveals transformation to diffuse large cell lymphoma in 30% to 50% of cases (Horning et al, 1984; Oviatt et al, 1984). Survival is then limited to months. The treatment of follicular NHL is discussed in Section 1.7.

The finding of recurrent, clonal cytogenetic abnormalities in acute leukaemia which were of prognostic significance (Yunis et al, 1984b) increased interest in the cytogenetic
analysis of the non-Hodgkin's lymphomas. It quickly became evident that karyotypic abnormalities in NHL were associated with morphologic subtypes of disease (Yunis et al, 1982), immunophenotype (Levine et al, 1986), sites of disease (Offit et al, 1989a) and survival (Yunis et al, 1987). The karyotypic analysis of NHL will now be discussed, with particular emphasis on follicular lymphoma.

1.3 Cytogenetics of follicular lymphoma

1.3.1 Background

The discovery of the Philadelphia chromosome in patients with chronic myeloid leukaemia demonstrated for the first time consistent chromosomal changes in human malignancy (Nowell et al, 1961). Cytogenetic analysis of the non-Hodgkin’s lymphomas began in 1962 but interpretation of the early results was hampered by poor banding techniques, inadequately analysed karyotypes with lots of unidentified marker chromosomes and lack of distinction between B-cell and T-cell disease (Rowley et al, 1980). In addition, at least three histological classifications were in use (Rappaport, 1966; Lukes et al, 1974; Gerard-Marchant et al, 1974) which made correlation of karyotype with morphology difficult. Despite these limitations, most lymphomas with analysable metaphases were found to have non-random clonal karyotypic abnormalities (Fukuhara et al, 1979; Rowley et al, 1980).
The 14q+ chromosome was established as the most common cytogenetic abnormality in NHL (Fukuhara et al, 1978a; 1978b; 1979; Rowley et al, 1980). The reciprocal translocation t(8;14)(q24;q32) was found to be almost pathognomonic for Burkitt’s lymphoma (Zech et al, 1976; Manolova et al, 1979). This suggested that, as with chronic myeloid leukaemia, specific cytogenetic abnormalities were associated with histologic subtypes of lymphoma.

In the early 1980s, several factors combined to establish a new era in lymphoma cytogenetics. The development of high resolution banding increased the number of bands per haplotype from 300 to 800 (Yunis et al, 1981) and synchronised culture techniques improved the number of analysable specimens from 70% to almost 100% (Yunis et al, 1982). The multiple histological classifications were unified into the Working Formulation (Rosenberg, 1982). Immunophenotyping to distinguish B-cells from T-cells became more common (Pinkus et al, 1979). Successful chromosomal analysis of over 1000 cases of NHL have now been reported (Heim et al, 1987). Clonal karyotypic abnormalities are present in at least 80-90%, probably more (Kaneko et al, 1983; Yunis et al, 1984a; Levine et al, 1985; Fifth Workshop, 1987). At least 17 recurrent translocations have been described in NHL (Bloomfield et al, 1988) and several other structural and numerical abnormalities. When the human homologue, c-myc,
of the avian myelocytomatosis virus oncogene, v-myc, was mapped to the segment of chromosome 8 involved in t(8;14)(q24;q32) of Burkitt's lymphoma (Dalla-Favera et al, 1982), a link was established between cytogenetic abnormalities and the molecular basis of lymphoma. It has been possible to correlate chromosomal abnormalities with histologic subtypes of lymphoma (Yunis et al, 1982; Bloomfield et al, 1983; Kaneko et al, 1983; Levine et al, 1985; Fifth Workshop, 1987; Koduru et al, 1987; Juneja et al, 1990) and with sites of disease (Offit et al, 1989a). More importantly, cytogenetic abnormalities may be of prognostic significance (Kaneko et al, 1983; Kristofferson et al, 1987; Levine et al, 1988; Schouten et al, 1990). Lastly, karyotypic analysis of lymphomas continues to identify new chromosomal abnormalities which may be of pathogenetic or prognostic significance (Levine et al, 1989; Offit et al, 1989b).

1.3.2 t(14;18) in follicular lymphoma

In the diverse histological group of poorly differentiated lymphomas, four of nine patients were found to have the reciprocal translocation t(14;18)(q32;q21) (Fukuhara et al, 1979). The nodular subgroup of this histologic type corresponds loosely to follicular lymphoma and in 1982 Yunis and coworkers confirmed the association of t(14;18) with follicular disease. The use of high resolution banding refined the breakpoints to 14q32.3 and 18q21.1 (Figure 1.5).
Figure 1.5  The reciprocal translocation  
t(14;18)(q32;q21)
The significant correlation of t(14;18) and follicular lymphoma was confirmed by other workers (Kaneko et al, 1983; Bloomfield et al, 1983; Levine et al, 1985; Speaks et al, 1987; Yunis et al, 1987; Koduru et al, 1987; Fifth Workshop, 1987; Offit et al, 1989a; Juneja et al, 1990; Schouten et al, 1990). The t(14;18) is now the single most common cytogenetic abnormality in non-Hodgkin's lymphoma and in one large series was found in 25% of patients (Fifth Workshop, 1987). It is found in 85% of cases of follicular lymphoma (Yunis et al, 1987) and in approximately 30% of diffuse disease (Yunis et al, 1982; Fifth Workshop, 1987; Juneja et al, 1990). The finding of t(14;18) as the sole abnormality in follicular lymphoma and its presence in untreated patients is highly suggestive of a causal role for this translocation (Yunis et al, 1987) but in some series over 50% of follicular lymphomas with karyotypic abnormalities lack this translocation (Bloomfield et al, 1983). Analysis of sequential karyotypes has strengthened the belief that t(14;18) is important in the pathogenesis of follicular lymphoma as it is retained in all subsequent analyses (Sanger et al, 1987; Levine et al, 1990).

1.3.3 Other cytogenetic abnormalities in follicular lymphoma

Multiple other recurrent, non-random abnormalities have also been described in follicular lymphoma, occurring in t(14;18) positive and t(14;18) negative disease; all can be present at diagnosis or may appear as the disease progresses.
(Yunis et al, 1982; 1987; Kaneko et al, 1983; Bloomfield et al, 1983; Levine et al, 1985; Speaks et al, 1987; Koduru et al, 1987; Fifth Workshop, 1987; Offit et al, 1989a; Juneja et al, 1990; Schouten et al, 1990). Table 1.2 gives details of the commonest of these abnormalities and the subtypes of follicular lymphoma with which they have been associated. Numerical and structural aberrations include trisomies 7, 8, 18 and 21 and deletions of chromosomes 2, 6 and 10.

There are multiple less specific abnormalities which also regularly occur in follicular lymphoma. Abnormalities of both arms of chromosomes 1 and 2, deletions of 13q32, and gains or losses of the sex chromosomes have been reported (Levine et al, 1985; Yunis et al, 1987; Fifth Workshop, 1987; Koduru et al, 1987; Speaks et al, 1987). The majority of these abnormalities occur in the presence of other cytogenetic defects and rarely occur alone (Yunis et al, 1987).

It must be stressed that all the karyotypic abnormalities discussed, including t(14;18), occur throughout all histologic subtypes of lymphoma and none is specific for follicular disease. Some occur in other solid tumours and leukaemia (Heim et al, 1987). In addition, lymphoma karyotypes usually contain many other clonal but not obviously recurrent defects which are too numerous to mention here (Mitelman, 1985).
Table 1.2  Cytogenetic abnormalities associated with subgroups of follicular lymphoma.  FL = follicular lymphoma(all groups); FSC = follicular small cell lymphoma; FMC = follicular mixed cell lymphoma; FLC = follicular large cell lymphoma.

<table>
<thead>
<tr>
<th>Abnormality</th>
<th>Subgroup</th>
<th>Reference</th>
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<tbody>
<tr>
<td>abnormalities of chromosome 3</td>
<td>FMC</td>
<td>Yunis et al, 1987</td>
</tr>
<tr>
<td></td>
<td>FLC</td>
<td>Yunis et al, 1987</td>
</tr>
<tr>
<td>del(2q)</td>
<td>FMC</td>
<td>Levine et al, 1985</td>
</tr>
<tr>
<td>del(6q)</td>
<td>FSC</td>
<td>Levine et al, 1985</td>
</tr>
<tr>
<td>del(6q), +7 or +12 or both</td>
<td>FMC, FLC</td>
<td>Yunis et al, 1987</td>
</tr>
<tr>
<td>+7</td>
<td>FLC</td>
<td>Levine et al, 1985</td>
</tr>
<tr>
<td></td>
<td>FL</td>
<td>Schouten et al, 1990</td>
</tr>
<tr>
<td>+8</td>
<td>FMC, FLC</td>
<td>Levine et al, 1985</td>
</tr>
<tr>
<td></td>
<td>FMC</td>
<td>Koduru et al, 1987</td>
</tr>
<tr>
<td>del(10q)</td>
<td>FSC, FMC</td>
<td>Speaks et al, 1987</td>
</tr>
<tr>
<td>+16</td>
<td>FL</td>
<td>Schouten et al, 1990</td>
</tr>
<tr>
<td>17q breaks</td>
<td>FLC</td>
<td>Koduru et al, 1987</td>
</tr>
<tr>
<td>+18</td>
<td>FSC, FMC</td>
<td>Speaks et al, 1987</td>
</tr>
<tr>
<td></td>
<td>FLC</td>
<td>Yunis et al, 1987</td>
</tr>
<tr>
<td></td>
<td>FL</td>
<td>Schouten et al, 1990</td>
</tr>
<tr>
<td>+21</td>
<td>FLC</td>
<td>Yunis et al, 1987</td>
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</table>
Yunis et al (1987) constructed a model based on their karyotypic findings in follicular lymphoma which suggested the primary abnormality in the majority of cases was t(14;18) and that both histologic subtypes and the behaviour of the disease were linked to the acquisition of further cytogenetic change. Evolution of follicular large cell lymphoma to high grade disease has been associated with the acquisition of t(8;14)(q24;q32) in addition to t(14;18)(Sham et al, 1989). Similar cytogenetic evolution has been demonstrated in the development of pre-B cell leukaemia from follicular lymphoma, with activation of c-myc (Gauwerky et al, 1988a; 1988b). Karyotypic complexity in the non-Hodgkin's lymphomas may be greater after treatment, reflecting continuing cytogenetic change as the disease progresses (Offit et al, 1991a). However, cytogenetic change does not always imply progression and histologic subtypes can change without any visible karyotypic alteration (Levine et al, 1990; Sanger et al, 1987).

1.3.4 Cytogenetics and prognosis

The finding of multiple non-random karyotypic abnormalities in patients with non-Hodgkin's lymphoma paralleled the discovery that most patients with acute leukaemia probably had cytogenetic defects (Yunis et al, 1981; 1984b) which defined subgroups of disease and were of prognostic significance (Yunis et al, 1984b; Bloomfield et al, 1986). In chronic lymphocytic leukaemia also, specific chromosomal
abnormalities have been correlated with survival (Juliusson et al, 1990). However, the relevance of cytogenetic abnormalities in predicting prognosis in NHL is still not established.

The cytogenetic analysis of NHL is in general more difficult than that of acute leukaemia. The karyotypes, even at diagnosis, are frequently more complex. Also, several histologic subgroups with different clinical features exist. Many of the published series which correlate karyotype with prognosis have been performed on small numbers of patients per histologic group (Kaneko et al, 1983; Kristoffersson et al, 1987) and have included patients studied both at diagnosis and relapse (Kaneko et al, 1983; Offit et al, 1991b). Larger series have restricted analysis to newly diagnosed cases only (Levine et al, 1988; Schouten et al, 1990) but are still hampered by the wide variation in age, stage and morphologic subgroup of the patients. Nevertheless, the established association of specific karyotypic abnormalities with the prognostically important histologic subgroups (Yunis et al, 1982; 1987) suggests that cytogenetics may partly predict survival.

It has been suggested that, as in acute myeloid leukaemia, the presence of karyotypically abnormal cells may be a prognostic indicator which is independent of histological sub-type in non-Hodgkin’s lymphoma.
Kristoffersson et al (1987) found that the greater the number of abnormal metaphases to normal metaphases, the shorter the survival. This was confirmed in follicular disease (Levine et al, 1988) but Schouten et al (1990) found no difference in survival between those with totally normal or abnormal karyotypes. More specific karyotypic abnormalities have been described as prognostic indicators in both diffuse and follicular lymphoma. In diffuse disease, breaks at 1q21-23, more than 4 marker chromosomes (Offit et al, 1991b), the presence of t(8;14)(q24;q32) (Kaneko et al, 1983) and abnormalities of chromosome 2 (Yunis et al, 1989) and 17 (Schouten et al, 1990) are all suggestive of aggressive lymphoma with shortened survival. In follicular lymphoma, the presence of t(14;18) as the sole abnormality has been correlated with follicular small cell disease and an indolent course with prolonged survival (Yunis et al, 1987). The presence of dup(3p) or trisomy 3 in follicular lymphoma has also been correlated with achievement of clinical remission and prolonged survival (Yunis et al, 1987). In contrast, those with t(14;18) and deletions of 13q32 quickly developed leukaemia and rapidly died (Yunis et al, 1987). The presence of t(14;18) in follicular mixed and large cell lymphoma has been correlated with failure to achieve remission and a poor prognosis (Yunis, 1989). Other possible poor prognostic features include trisomy 2 or dup(2p), the presence of homogeneously staining regions or double minutes.
(Yunis et al, 1987) and abnormalities of chromosome 17 (Levine et al, 1988).

Many of these associations have not been confirmed or have been refuted by other groups. Offit et al (1991b) found no correlation between chromosomal abnormalities and survival in low grade non-Hodgkin’s lymphoma, although median survival was only 2 years. Another study found no difference in survival found between those with karyotypically normal cells only and those with +3, +12, del(6q), iso(17q) or t(14;18) (Kristoffersson et al, 1987). However, larger studies are required to confirm or refute some of these findings.

1.4 Oncogenes and follicular lymphoma

1.4.1 Background

The discovery of viral oncogenes - genes capable of inducing malignant tumours in animals - and their human counterparts was a major advance in the understanding of the genetic events in neoplasia (Bishop, 1985). Klein (1981) had already shown that murine immunoglobulin genes were implicated in the development of experimental tumours in mice. In Burkitt’s lymphoma, c-myc, the human equivalent of v-myc, the gene causing avian myelocytomatosis, translocates from chromosome 8q to 14q (Dalla-Favera et al, 1982), the site of the immunoglobulin heavy chain locus (Croce et al, 1979).
The variant translocations t(2;8) and t(8;22) result in the juxtaposition of c-myc with the immunoglobulin light chain loci (Croce et al, 1985; 1986). The transcription of the translocated c-myc is markedly increased by genetic elements in the three immunoglobulin gene loci which are capable of acting over considerable chromosomal distances (Croce et al, 1985). C-myc encodes for a DNA-binding protein and it has been postulated that its deregulation may drive cells to divide continuously without subsequent differentiation (Klein et al, 1986).

The immunoglobulin heavy chain locus is also the site of two other translocations commonly found in non-Hodgkin’s lymphoma. The t(11;14)(q13;q32) translocation has been described in diffuse small and large cell lymphomas and in chronic lymphatic leukaemia (Yunis et al, 1982). Cloning of the breakpoints of t(11;14)(q13;q32) from a patient with chronic lymphatic leukaemia led to the identification of a putative proto-oncogene, B-cell lymphoma/leukaemia 1 (bcl-1) at 11q13 (Erikson et al, 1984; Tsujimoto et al, 1984b) although the gene has still to be characterised and its oncogenic potential demonstrated.

The t(14;18)(q32;q21) is found in 85% of follicular lymphomas (Yunis et al, 1987) and 30% of diffuse lymphomas (Juneja et al, 1990). The proto-oncogene bcl-2 at chromosome 18q21 was identified after cloning of the breakpoints of
t(14;18) in a cell line derived from a patient with acute B-cell leukaemia (Tsujimoto et al, 1984a; Bakhshi et al, 1985). This gene has been sequenced (Seto et al, 1988) and its malignant potential demonstrated by gene transfer (Reed et al, 1988). The discovery of bcl-2 is interesting for several reasons. Firstly, unlike c-myc, it has no known viral homologue and was identified by cloning of a translocation breakpoint (Tsujimoto et al, 1984a; Bakhshi et al, 1985; Cleary et al, 1985). Secondly, the normal immunoglobulin gene rearrangements during B-cell maturation may provide a mechanism for the t(14;18) translocation (Tsujimoto et al, 1985a; 1988). Lastly, it may function as a new class of oncogene, preventing cell death and promoting survival without proliferation (Hockenbery et al, 1990). The molecular detection of rearrangements of bcl-2 in follicular lymphoma forms an integral part of this thesis. The structure and function of bcl-2 will therefore be discussed in detail.

1.4.2 Bcl-2 oncogene - structure and function

The role of the bcl-2 gene in follicular lymphoma has been recently reviewed (Cotter, 1990). The t(14;18) breakpoints were first cloned from a B cell leukaemia cell line with both t(14;18) and t(8;14). Using immunoglobulin heavy chain probes and a "chromosome walking" technique translocated sequences were cloned from 18q21. Probes derived from these sequences detected rearrangements of
homologous DNA in the leukaemic cell line and in a follicular lymphoma with t(14;18). A putative oncogene, bcl-2 (B cell leukaemia/lymphoma 2) was identified on chromosome 18q21 (Tsujimoto et al, 1984a). Its rearrangement in follicular lymphoma with t(14;18) was confirmed by other workers (Bakhshi et al, 1985; Cleary et al, 1985).

The normal bcl-2 gene has three exons (Seto et al, 1988). The first is large and untranslated and the second contains classic promoter sites and a decanucleotide sequence homologous with the immunoglobulin variable enhancer (Seto et al, 1988). The gene is transcribed into three overlapping mRNAs which encode for two proteins, depending on the splice site used (Tsujimoto et al, 1986). Bcl-2-alpha protein and bcl-2-beta protein are identical for the first 196 amino acids but differ in their carboxyl terminus. The alpha protein is 239 amino acids long and has a mass of 26 kDaltons (Tsujimoto et al, 1986). It bears some homology to a predicted Epstein-Barr virus protein, BHRF-1 (Cleary et al, 1986a). The beta protein contains 205 amino acids and has a mass of 22 kDaltons (Tsujimoto et al, 1986). The role of bcl-2 protein in normal and neoplastic cells is not yet known. The protein has no kinase activity (Cleary et al, 1989) and is not a GTP binding protein (Monica et al, 1990) as previously thought (Haldar et al, 1989).
The exact cellular location of bcl-2 protein is also in doubt. Subcellular localisation of the protein in a cell line bearing t(14;18) suggested association of the protein with both nuclear and cytoplasmic membranes; immunofluorescent studies showed that most staining was confined to the perinuclear region, consistent with location in the rough endoplasmic reticulum (Chen-Levy et al, 1989; Cleary et al, 1989). However, Hockenbery et al (1990) localised bcl-2 protein to the inner mitochondrial membrane.

Much of the presumed function of bcl-2 has been gained from in vitro cell culture and from transgenic mice. The expression of the bcl-2 gene has been extensively studied in both normal and neoplastic cells. Bcl-2 transcript RNAs are high during pre-B cell development but expression is downregulated as the cell matures (Graninger et al, 1987). Within mouse B-cell lines, bcl-2 is expressed in pre-B cells and more mature B-cells but not pro-B cells or plasmacytoid cells (Gurfinkel et al, 1987). This suggests a role for bcl-2 at the pre-B cell stage of normal B-cell maturation. The gene is also transcribed when normal B and T lymphocytes are stimulated with the appropriate mitogens (Reed et al, 1987). B cell lymphomas with t(14;18) have highly increased levels of bcl-2 mRNA and bcl-2 protein, indicating marked deregulation of the translocated allele; the normal allele is
transcriptionally silent (Cleary et al., 1986a; Tsujimoto et al., 1986; Seto et al., 1988).

The identification of bcl-2 as a possible oncogene was unique as cellular oncogenes have been previously identified by homology to their viral counterparts and not by cloning of translocation breakpoints. It was therefore necessary to demonstrate the oncogenic potential of bcl-2 both in vitro and in vivo. Transfection of bcl-2 into fibroblast cell lines conferred a growth advantage to the cells in vitro but did not induce transformation (Reed et al., 1988). Deregulated bcl-2 expression enhanced the growth of a human lymphoblastoid cell line in vitro, permitting it to grow at lower cell densities and reduced serum concentrations (Reed et al., 1989). Similarly, the introduction of bcl-2 cDNA into interleukin-3 dependent lymphoblastoid cell lines resulted in less dependency on growth factors and prolonged survival of cells in G₀ but not proliferation (Vaux et al., 1988; Nunez et al., 1990). When the transfected cells were injected into mice, they produced tumours but only after a latent period of four weeks.

Cell lines expressing both c-myc and bcl-2 have demonstrated increased frequency and decreased latency in tumour production when injected into immunodeficient mice, suggesting cooperation between the two genes (Nunez et al., 1989). Transgenic mice with bcl-2/Ig fusion genes
inserted into their germline developed regional lymphadenopathy and an expanded follicle centre cell population but initially remained healthy (McDonnell et al, 1989). However, after several months, 11% of the transgenic mice developed high grade lymphomas. The most common type was diffuse large cell immunoblastic lymphoma and 50% of these showed rearrangements of c-myc (McDonnell et al, 1991). Doubly transgenic mice which constitutively express both bcl-2 and c-myc showed hyperproliferation of pre-B and B-cells and developed tumours more rapidly than those which expressed c-myc alone (Strasser et al, 1990).

Thus it appears that deregulation of bcl-2 in vitro and in transgenic mice confers a growth advantage to B-cells without rendering them overtly tumourigenic. After a latent period, secondary molecular events such as activation of c-myc lead to lymphomagenesis. This parallels the history of follicular lymphoma in man, where those with t(14;18) alone have an indolent course and may not require treatment for many years (Yunis et al, 1987). The prolonged survival of t(14;18) B-cells may render them more susceptible to further cytogenetic change with the eventual development of a highly malignant clone. Both t(14;18) and t(8;14) have been described in high grade lymphoma (Thangavelu et al, 1990). The histologic and clinical progression of follicular lymphoma to high grade disease or pre-B cell leukaemia with
initial rearrangement of bcl-2 and subsequent rearrangement of c-myc has also been described (de Jong et al, 1988; Gauwerky et al, 1988a; 1988b; Lee et al, 1989).

Doubts have been cast on the continued importance of bcl-2 in lymphomagenesis after the development of secondary cytogenetic abnormalities. Kiem et al (1990) analysed a cell line derived from a patient with lymphoblastic lymphoma and revealed a three way translocation involving chromosomes 8, 14 and 18. Molecular studies showed that c-myc had translocated to the der(14) already bearing bcl-2. Bcl-2 was displaced and inactivated but c-myc was overexpressed, suggesting that continued activation of bcl-2 may not be necessary in the sequential development of high grade lymphoma.

The mechanism of how deregulated bcl-2 prolongs B cell survival is not yet known. Within normal lymphoid follicles, those B cells not destined to become memory or plasma cells undergo apoptotic cell death. It has been proposed that bcl-2 may block this programmed cell death without promoting cell division (Hockenbery et al, 1990).

1.4.3 Mechanism of t(14;18) translocation

As discussed, there are three recurrent translocations in B cell lymphoma which involve the immunoglobulin heavy
chain locus at 14q32 - t(14;18), t(11;14) (Tsujimoto et al,1984a;1984b) and t(8;14) (Dalla-Favera et al,1982). It has been suggested that the recombinase enzyme which is responsible for VDJ joining in normal B cells may have a role in t(11;14) and t(14;18) (Tsujimoto et al,1985a;1988). This enzyme recognises highly conserved heptamer/nonamer signalling sequences between V,D and J regions (Alt et al,1987). Flanking spacer regions of 12 or 23 nucleotides prevent aberrant joining. Similar heptamer/nonamer signalling sequences have been described close to the breakpoint sites of bcl-2 on 18q21 (Tsujimoto et al,1988) suggesting that mistakes may be made in VDJ joining and bcl-2 is inserted instead of a V region. However, the presence of these sequences has been refuted by others (Bakhshi et al,1985; Cleary et al,1985). Sequence analysis of the breakpoints of t(14;18) has revealed the presence of extra nucleotides reminiscent of N regions (Roth et al,1989) which are inserted by terminal deoxynucleotidyl transferase (Desiderio et al,1984) between VD and DJ regions during normal recombination (Cleary et al,1985; Bakhshi et al,1985;1987). Bakhshi and others (1987) have proposed that a double stranded break in bcl-2 and an immunoglobulin endonuclease mediated break in the heavy chain locus may be repaired aberrantly after N segment insertion. The translocation is not fully reciprocal as DNA is lost between the D and J regions, resulting in the D region being fused to the remnant of bcl-2 left on 18q21
The immunoglobulin enhancer region between the JH and switch regions is retained in t(14;18) (Bakhshi et al, 1985; Tsujimoto et al, 1984a) but would have to function over 50kb of DNA to influence bcl-2 (Cleary et al, 1986a).

1.5 Molecular detection of bcl-2 rearrangements in follicular lymphoma

1.5.1 Detection by conventional electrophoresis

In 85% of follicular lymphomas with t(14;18), the breakpoints on 18q21 cluster within two very short breakpoint regions. The major breakpoint region (mbr) is approximately 150 bases long and lies within the large untranslated 3' exon; 60% of breaks are contained within the mbr (Bakhshi et al, 1985; Cleary et al, 1985; Tsujimoto et al, 1985b; Weiss et al, 1987). The minor cluster region (mcr) lies at least 20kb 3' of the mbr and contains a further 25% of breakpoints (Cleary et al, 1986b). Occasionally, breaks may occur 5' of the bcl-2 gene with inversion of the gene prior to translocation (Tsujimoto et al, 1987). This leaves approximately 10% of breakpoints on 18q21 falling outwith the three regions. The breakpoints on chromosome 14 lie within the JH region of the immunoglobulin heavy chain locus (Tsujimoto et al, 1984a).

The clustering of breakpoints within bcl-2 at the mbr and mcr and also within the JH region of the immunoglobulin
heavy chain locus allows detection of t(14;18) by conventional electrophoresis using the method of Southern (1975). Using recombinant DNA probes derived from the major breakpoint region of bcl-2, rearrangements of the gene have been found in approximately 60% of follicular lymphomas and 30% of diffuse large cell lymphomas (Lee et al, 1987b; Lipford et al, 1987; Aisenberg et al, 1988). Weiss et al (1987) used probes covering the major, minor and 5' breakpoint regions and detected bcl-2 rearrangements in virtually all follicular lymphomas and almost 30% of diffuse disease. Rearrangements of bcl-2 have been found in 57% of Hong-Kong Chinese with follicular lymphoma (Loke et al, 1990) but in only 33% of Japanese follicular lymphomas, suggesting possible racial differences in the incidence of the translocation (Amakawa et al, 1989). Detection of t(14;18) by molecular methods has some advantages over conventional cytogenetics. Conventional electrophoresis has revealed the translocation in those in whom cytogenetics has failed (Lee et al, 1987b; Chaganti et al, 1989) and has also detected submicroscopic rearrangements of bcl-2 (Lee et al, 1987b). The breakpoints are also remarkably conserved, with JH;18q21 rearrangements remaining unchanged for several years despite clonal variations in the untranslocated heavy chain allele (Raffeld et al, 1987).
1.5.2 Detection by the polymerase chain reaction

The translocation t(14;18) is also suitable for enzymatic amplification by the polymerase chain reaction (Saiki et al, 1988). Using a consensus primer for all the JH regions and an oligonucleotide complementary to DNA sequences around the mbr of bcl-2, Lee et al (1987a) specifically amplified t(14;18) in DNA from a patient with follicular lymphoma and cytogenetic evidence of the translocation. The technique was so sensitive that one cell with the translocation could be detected at a dilution of 1:100,000. Only one microgram of genomic DNA was required for each reaction. Similarly, sequences from the mcr have been amplified by PCR (Ngan et al, 1989). The extreme sensitivity of PCR has made it a suitable technique for detecting occult t(14;18) bearing cells in tissue thought histologically and molecularly (by Southern blotting) to be uninvolved with lymphoma (Crescenzi et al, 1988; Stetler-Stevenson et al, 1988). Detection of t(14;18) by PCR has also demonstrated dissemination in gastric lymphoma (Cunningham et al, 1989) and has confirmed extranodal disease in follicular lymphoma (Price et al, 1989). The technique is also useful for detecting t(14;18) in paraffin embedded tissue which is otherwise unsuitable for molecular analysis (Pezzella et al, 1989).
The use of PCR to detect t(14;18) bearing cells has also produced some startling results. The translocation has been detected in non-malignant tonsils and in B-cells purified from the blood of normal donors, suggesting that t(14;18) may occur in the absence of lymphoid malignancy (Limpens et al, 1990). Also surprisingly, t(14;18) was detected by PCR in carefully controlled experiments in over 30% of lymph nodes from patients with Hodgkin's disease (Stetler-Stevenson et al, 1990). No karyotypic data was available from these cases, but such results are at variance with the known cytogenetics of Hodgkin's disease. In the largest recent series of karyotypes, no cases of t(14;18) were detected amongst 31 patients with Hodgkin's disease but three patients had 14q+ chromosomes of unknown origin (Tilly et al, 1991). The results of Stetler-Stevenson and her colleagues remain unique (Cleary et al, 1990).

1.6 Immunohistochemical detection of bcl-2 protein

The over-expression of normal bcl-2 protein caused by deregulation of bcl-2 in cells with t(14;18) can be detected by immunohistochemical techniques (Figure 1.6). Using monospecific antibodies raised to the protein, Ngan et al (1988) examined frozen sections of normal and neoplastic nodes. Cells staining positively for bcl-2 protein were found in follicles and interfollicular spaces of malignant nodes but bcl-2 protein was not detected in non-neoplastic...
Figure 1.6  Follicular non-Hodgkin's lymphoma. Case 10. Immunocytochemical detection of bcl-2 protein. x 100.
nodes. Every tumour with t(14;18), irrespective of breakpoints, expressed detectable bcl-2 protein. It was also detected in 30% of diffuse lymphomas, some of which were of follicle cell origin. This figure approximates to the cytogenetic incidence of t(14;18) in diffuse disease. However, four lymphomas which lacked the t(14;18) translocation also expressed bcl-2 protein. The expression of bcl-2 protein in the absence of t(14;18) was also confirmed by others (Pezzella et al, 1990b).

Although it was hoped that immunohistochemical detection of bcl-2 protein in lymph node sections would be a useful diagnostic marker for B-cell lymphomas, this may not be the case (Pezzella et al, 1990c). These workers confirmed that bcl-2 protein was detectable in the neoplastic germinal centres of follicular lymphomas and not in normal centres, but they also detected bcl-2 protein in normal B and T-lymphocytes and in other lymphoid disorders such as hairy cell leukaemia which lacked the t(14;18). This discrepancy may be due to staining techniques (Pezzella et al, 1990c) but clearly the expression of bcl-2 protein in lymphoid malignancies requires further investigation.

1.7 Treatment of follicular lymphoma

Treatment of follicular non-Hodgkin’s lymphoma for the majority of patients is not curative but aims to relieve
symptoms of disease. The natural history of these indolent lymphomas suggests that in many cases treatment can be delayed for several years without detrimental effects (Horning et al, 1984). In their series of eighty three patients with initially untreated low grade lymphoma (including twenty one with small cell lymphocytic lymphoma) survival was 82% at five years and 73% at ten years. The median time from diagnosis to treatment was three years. Therapy is usually commenced because of evidence of disease progression or development of systemic symptoms, anaemia or thrombocytopenia. The type of treatment is based on both age of the patient and stage of disease, usually determined by standard morphological and immunophenotyping techniques. Localised Stage 1 disease is rare in the follicular lymphomas but approximately 50% may be cured by radiotherapy alone (Paryani et al, 1983). Chemotherapy plus radiotherapy is given to those with more advanced lymphoma. Overall disease-free survival may approach 52% at five years despite disseminated lymphoma at diagnosis (McLaughlin et al, 1987). Single agent therapy such as chlorambucil can produce complete responses in one third to three quarters of patients (Hoppe et al, 1981) and may be as effective as a combination of drugs. It has already been shown in diffuse lymphomas that drug regimes with increased dose intensity are capable of producing higher and more sustained complete responses (Klimo et al, 1985). In follicular lymphoma, the role of aggressive drug treatment
is still being evaluated and although initial responses may appear more encouraging, overall survival is not yet increased (Young et al, 1988). There is however a small group of patients with follicular lymphoma, often young, with poor prognostic features at diagnosis and progressive disease. Treatment with single agent chemotherapy may fail and median survival is only two years (Gallagher et al, 1987). New approaches to the treatment of such aggressive refractory non-Hodgkin’s lymphoma are constantly being devised. Limited success has been reported using anti-idiotype antibodies derived from mice which react uniquely against the patients’ tumour cells (Meeker et al, 1985). Other, less experimental approaches have included alpha interferon which is capable of inducing responses in 40% to 50% of patients treated, although the response is often partial and short-lived (Vander Molen et al, 1990). Interferon may be more useful as adjuvant therapy in combination with chemotherapy regimes (Steis et al, 1987). The role of bone marrow transplantation in patients with lymphoma has been reviewed by Armitage (1989). The majority of patients with lymphoma lack a suitable allogeneic or syngeneic donor and the majority of transplants have been autologous. Durable responses have been produced in poor prognostic patients with intermediate or high grade disease by a combination of high dose chemotherapy and autologous transplantation (Philip et al, 1985). The procedure is not so successful in those with
refractory lymphoma (Verdonck et al, 1987). However, the incidence of marrow involvement in low grade lymphoma, even after treatment, is normally a contraindication to autologous transplant. Monoclonal antibodies against tumour cell surface markers such as CD19 and CD20 have been used to purge autologous marrow from patients with low grade lymphoma prior to reinfusion (Freedman et al, 1991). Those who had entered complete remission before transplant did better than those who still had residual disease. Some of the problems associated with re-infusing contaminated marrow can be overcome by using autologous peripheral blood stem cells (Bell et al, 1986; Juttner et al, 1989; Lasky, 1989; Kessinger et al, 1991) and it is likely that this technique will be utilised more often for those whose marrow is otherwise unsuitable.

1.8 Aims of thesis

The finding of consistent and specific cytogenetic abnormalities has contributed to our understanding of the pathogenesis of malignant lymphoma. Several oncogenes have been mapped to translocation breakpoints and the role of their protein products in carcinogenesis established (Buckley, 1988; Stanbridge et al, 1990). The stepwise model of carcinogenesis proposed by Yunis et al, (1987) has been supported by sequential karyotypic analysis of lymphomas. Clonal karyotypic changes are of prognostic and diagnostic
significance in acute leukaemia although the role of cytogenetics in the management of non-Hodgkin’s lymphoma is not yet established. However, the presence of t(14;18) in over 50% of patients with follicular lymphoma provides both a cytogenetic and molecular marker of disease. As treatment strategies become more aggressive, accurate staging at diagnosis and the detection of minimal residual disease and early relapse will become more important. The frequency of t(14;18) in follicular lymphoma and the ease with which it can be detected using sensitive molecular methods make it an ideal marker of disease activity.

This thesis aims firstly, using cytogenetics, conventional electrophoresis and DNA amplification by the polymerase chain reaction, to determine the incidence of t(14;18)(q32;q21) in a Scottish population of 72 patients with follicular lymphoma. The majority of published series have used either karyotopic (Speaks et al, 1987; Yunis et al, 1987) or molecular analysis alone (Weiss et al, 1987; Pezzella et al, 1990a), with considerable variation in incidence of the translocation. This thesis therefore forms one of the largest series of patients with follicular lymphoma studied simultaneously by all three techniques.

Secondly, the ability of cytogenetic and molecular methods to detect t(14;18) in diagnostic material will be assessed and the limitations of each technique will be
discussed. It might be anticipated that the increased sensitivity and specificity of molecular detection of t(14;18) would relegate cytogenetic analysis to second place (Chaganti et al, 1989).

Thirdly, molecular detection of t(14;18) is used to determine the presence of occult lymphoma in staging marrows, after treatment and in peripheral blood stem cell harvests.

Finally, the relevance of the multitude of other karyotypic abnormalities found on cytogenetic analysis of the 72 cases is also discussed.
CHAPTER 2

MATERIALS AND METHODS
MATERIALS AND METHODS

The work described in this thesis was performed while I was employed as Clinical Scientist in the Cytogenetics Section of the MRC Human Genetics Unit, Edinburgh. During this time, I became proficient in all aspects of the cytogenetic techniques described below, including karyotypic analysis of NHL and other haematological malignancies. However, the majority of karyotypic analyses in this thesis were performed by Dr Fiona Ross, and the remainder by Dr Lynn Hendry and myself. All the molecular work, described in Sections 2.3 to 2.10, was carried out by myself. For the majority of techniques used in this thesis, I am indebted to Maniatias, Fritsch and Sambrook (1982) for their excellent practicality.

2.1 Collection of patients’ specimens

Lymph nodes and other tumour biopsies, bone marrows and occasionally peripheral blood were collected as part of routine diagnostic and staging procedures from patients with non-Hodgkin’s lymphoma. Fresh material was set up for karyotypic analysis. Any residual material was frozen as chunks or cell pellets at -70°C or in liquid nitrogen for subsequent molecular analysis. Additional samples of lymph nodes for DNA extraction were obtained from frozen section blocks stored at -70°C in the Department of Pathology.
2.2 Karyotypic analysis

2.2.1 Materials

Culture medium

RPMI 1640
20–30% fetal calf serum
2mM glutamine

Wright's stain

1. Mix 1.25g of Wright's stain (Sigma) slowly with 500ml of methanol and stir for 60 minutes.

2. Filter 0.25% solution through double thickness Whatman No.1 paper into dark bottle and store at room temperature in the dark for 14 days.

Phosphate buffer

Prepare 0.06M phosphate buffer by mixing 0.06M KH₂PO₄ solution and 0.06M Na₂HPO₄ solution in a ratio of 51:49 (pH6.8)

2.2.2 Method

1. Make cell suspension from lymph nodes by agitating vigorously with sterile orange sticks in warmed medium.

2. Perform nucleated cell count of bone marrow or lymph node suspension using methylene blue.

3. Set up cultures with 1x10⁶ nucleated cells/ml in 5-10ml of medium.

4. Place in gassed incubator (5% CO₂) at 37°C.

5. Direct and 24hr cultures: add 0.1µg/ml deacetylmethylcolchine (Colcemid) (Sigma) 45 minutes prior to harvesting.

6. Synchronised cultures: block with fluorodeoxyuridine
(FUDR) (Sigma) and uridine (Sigma) to final concentrations of 10^{-7} M and 4 \times 10^{-6} M respectively. After 17 hours, release with 10^{-3} M thymidine (BDH), culture for further 5-7 hours and add Colcemid as before 10 minutes prior to harvest.

7. To harvest, centrifuge at 600g, 15% brake for 8 minutes.

8. Remove supernatant by gentle suction, resuspend cells and add 10ml of 0.075M potassium chloride solution.

9. Incubate at 37°C for 25-30 minutes and centrifuge as before.

10. After removing supernatant, fix cells in methanol:acetic acid 3:1(v:v); add first ml while agitating cells.

11. Store at 4°C overnight before changing fix 4 times.

12. Adjust cell concentration before dropping onto clean slides. Incubate slides in oven at 60°C overnight to age.


14. Wash with tap water for 5 seconds and dry immediately with hot air blower. Adjust timing of stain according to trial slide.

15. Mount coverslip with DPX (BDH).
2.3 Extraction of genomic DNA

2.3.1 Materials

Resuspension buffer

150mM NaCl
10mM Tris.Cl, pH7.5
10mm EDTA
0.5% SDS

TE solution

10mM Tris.Cl, pH7.4
1mm EDTA, pH8.0

RNAase

Pancreatic RNAase (Sigma) was dissolved in 10mM Tris.Cl (pH7.5), 15mM NaCl at a concentration of 10mg/ml. The solution was heated to 100°C for 15 minutes, cooled to room temperature and dispensed into aliquots for storage at -20°C.

Proteinase K

A stock solution of proteinase K (Boehringer Mannheim) was prepared at a concentration of 20mg/ml in H₂O. Aliquots were stored at -20°C.

2.3.2 Method

1. Thaw frozen cells or lymph node chunks on ice. Chop lymph nodes with sterile scalpels. Wash cells once with phosphate buffered saline (PBS).

2. In a polypropelene tube with plastic cap, resuspend cells in 0.5-10ml of resuspension buffer according to cell numbers.

3. Add 50ug/ml of RNAase and incubate at 37°C for
60 minutes.
4. Add 100μg/ml of proteinase K and leave at 37°C for 4-24 hours.
5. Mix thoroughly with equal volume of water-saturated phenol until emulsion forms.
6. Centrifuge at 1600g at 4°C for 10 minutes.
7. Remove aqueous phase and repeat steps 5 and 6 once more.
Repeat step 8 until no protein is visible at interface.
9. Extract aqueous phase twice more with equal volumes of chloroform:isoamylalcohol(24:1).
10. Add 0.5 volume of 7.5M ammonium acetate to aqueous phase, mix and then add 2 volumes of ice-cold ethanol.
11. Spool DNA onto clean glass rod and allow to air dry.
12. Rinse in 70% ethanol and allow to dissolve in 0.1-1ml of TE solution in sterile microfuge tubes. Dissolution can be aided by gentle rotation. Store at 4°C.
13. The same method can be used to extract small amounts of DNA from less than 10⁶ cells. Microfuge tubes and scaled-down volumes are required. Minute quantities of DNA can be precipitated by addition of ethanol, then centrifuged and vacuum desiccated before dissolution in 50-100ul of TE solution.
2.4 Estimation of DNA concentration

DNA concentration was estimated by spectrophotometer. A small aliquot was diluted in distilled water and the optical density at 260nm and 280nm was measured. An OD\textsubscript{260} of 1 corresponds to approximately 50\textmu g/ml of double-stranded DNA. The ratio between OD\textsubscript{260} and OD\textsubscript{280} gave an estimate of purity, being 1.8 in pure preparations.

2.5 Digestion of DNA with restriction enzymes

2.5.1 Materials

Dithiothreitol (DTT)
For 1M stock solution, dissolve 3.09g of DTT (Boehringer Mannheim) in 20ml of 0.01M sodium acetate (pH 5.2). Sterilise by filtration and dispense into aliquots of 1ml. Store at -20°C.

Spermidine
Make up 1M stock solution of spermidine (Sigma) and store in aliquots at -20°C.

2.5.2 Method

1. In a microfuge tube, add 10.5\textmu g of purified genomic DNA to distilled water to 80ul. Mix and leave at 37°C for 30 minutes.

2. Add 10ul of appropriate 10x restriction enzyme digestion buffer (Boehringer Mannheim). Add spermidine and DTT to final concentration of 0.025M each and 40 units of restriction enzyme (Boehringer Mannheim)
(total digest volume 105.25ul). Mix and leave overnight at appropriate temperature.

3. To ensure complete digestion, 5.25ul of each sample can be run on 0.8% agarose gel and stained (Section 2.6). Adequately digested samples are frozen at -20°C until required.

4. To prepare digests for gel electrophoresis, thaw at room temperature and adjust monovalent cations to final concentration of 0.2M by adding 5M NaCl (if low salt buffer used). Add 2x volume of cold ethanol.

5. Leave at -70°C for 1 hour, centrifuge for 20 minutes at 12000g at 4°C in microfuge and remove supernatant.

6. Resuspend DNA pellet in 100ul of 70% ethanol, leave at -20°C for 1 hour and repeat centrifugation.

7. Remove supernatant and dry pellet by vacuum desiccation for 10 minutes. Resuspend in 30ul of TE buffer.

2.6 Agarose gel electrophoresis

2.6.1 Materials

20x TAE

0.4M Tris.HCl
0.2M sodium acetate
0.01M EDTA

10x gel loading buffer

0.25% bromophenol blue (Sigma)
0.25% xylene cyanol (Sigma)
25% Ficoll (type 400) (Sigma)

Store at room temperature
Denaturing solution
1.5M NaCl
0.5N NaOH

Neutralising solution
1M Tris.HCl (pH7.4)
2M NaCl

2.6.2 Method
1. Seal open ends of clean, dry electrophoresis tray with autoclave tape. Insert comb at one end.
2. Prepare 23cm x 23cm 0.8% agarose gel by dissolving 4g of agarose (Sigma) in 500ml of 1x TAE. Heat solution in microwave to aid dissolution and cool to 60°C while stirring.
3. Pour agarose into sealed tray and allow to set. Remove comb carefully and mount gel in electrophoresis tank. Cover with 1x TAE to a depth of 1mm.
4. Before loading on gel, mix thawed digested DNA samples with 5ul of 10x gel loading buffer. Load samples and appropriately sized DNA markers and electrophorese at 2V/cm for 16-24 hours. The gel can be kept cool by placing in cold room at 4°C.
5. Stain gel in dH2O containing 0.5ug/ml of ethidium bromide for 30 minutes and photograph under ultraviolet light (302nm) using Kodak TMAX 100 Professional film. Destain for 10 minutes in dH2O.
6. Depurinate gel in 500ml of 0.25M HCl for 20 minutes.
7. Immerse gel in 500ml of denaturing solution for
45 minutes. Agitate gently on rotator platform.

8. Rinse briefly in distilled water and then add 500ml of neutraliser solution. Agitate for 30 minutes. The gel is now ready for Southern blotting.

2.7 Southern blotting (Southern, 1975)

2.7.1 Materials

20x SSC
3.0M NaCl
0.3M tri-sodium citrate

2.7.2 Method

1. Prepare blotting tank by placing piece of No1 Whatman paper over clean glass sheet and allowing ends to soak in tank containing 20x SSC. Place second piece of filter paper cut to size of gel on top. Allow both to become fully saturated.

2. Rinse gel briefly in 2x SSC and place carefully on filter paper.

3. Cover with same size piece of Hybond-N Plus (Amersham) hybridisation transfer membrane which has been soaked in 2x SSC. Carefully remove any air bubbles between gel and membrane.

4. Surround gel with Saran wrap or Parafilm to prevent liquid flowing directly from tank to absorbent paper.

5. Place 3 further sheets of filter paper soaked in 2x SSC on top, and then absorbent paper towels and a 500g weight.
6. Blot for 16-24 hours, replacing paper towels when they become saturated.
7. Remove towels and filter paper and mark gel slots on transfer membrane with ballpoint pen. Cut off one corner to allow orientation. Wash Hybond-N Plus filter in 2x SSC and air dry. Bake for 1 hour at 80°C and then bind DNA to filter by exposing DNA side to ultraviolet light at 302nm for 3 minutes.
7. Store at 4°C.

2.8 Radioactive labelling of DNA probes

2.8.1 Materials

Two chromosome 18 derived genomic DNA probes were used to detect t(14;18) by Southern blotting and are shown diagramatically in Figure 2.1.

Figure 2.1 Chromosome 18 derived probes used to detect t(14;18) by Southern blotting. The bcl-2 probe detects breakpoints falling at the major breakpoint region (mbr) and the pfl-2 probe at the minor cluster region (mcr).

The bcl-2 probe, (a gift from Dr Y Tsujimoto), is a 3.5kb EcoRI-HindIII fragment of chromosome 18 cloned into pSP65 vector and detects breakpoints at the major breakpoint region (Tsujimoto et al,1985b). The pfl-2 probe, (a gift from
Dr ML Cleary) is a 4kb EcoRl fragment of chromosome 18 inserted into the EcoRl site of vector pBR 322 and detects rearrangements at the minor cluster region (Cleary et al, 1986b). Although a probe to the 5' region of bcl-2 was available (Weiss et al, 1987) suitable stringency conditions were not achieved and use of this probe was abandoned. To complement detection of bcl-2 rearrangements by chromosome 18 probes, filters were stripped and rehybridised with a 2.5kb EcoRl fragment of the immunoglobulin heavy chain joining region from chromosome 14 (Flanagan et al, 1982).

2.8.2 Method

Probes were radioactively labelled by the hexamer random priming technique (Feinberg et al, 1983) using a Random Priming Labeling Kit (Boehringer Mannheim). The double-stranded vectors containing the probes were linearised with the appropriate restriction enzyme and concentration of each estimated by comparison with known concentrations of lambda markers. Fifty nanograms of each probe was used per labelling reaction.

1. Add distilled water to 50ng of probe to volume of 6ul. Boil for 10 minutes and cool in ice for 10 minutes.

2. Add 1ul each of unlabelled nucleotides dATP, dGTP and dTTP (0.5mM/l).

3. Add 2ul of 10x reaction mixture and mix thoroughly.
4. Add 50uCi of P³²-labelled dCTP (Amersham)(specific activity > 3000Ci/mM), mix and then add 1ul (5 units) of Klenow enzyme. Mix and leave overnight at room temperature or incubate for 1 hour at 37°C.

2.8.3 Determination of activity

Removal of unlabelled nucleotides was achieved using Sephadex G 50 DNA grade NICK columns (Pharmacia).

2.8.3.1 Materials

TNE

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Component</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>10mM</td>
<td>Tris.HCl</td>
<td>8.0</td>
</tr>
<tr>
<td>100mM</td>
<td>NaCl</td>
<td></td>
</tr>
<tr>
<td>1mM</td>
<td>EDTA</td>
<td>8.0</td>
</tr>
</tbody>
</table>

2.8.3.2 Method

1. Add 80ul of distilled water to labelling mix to give total volume of 100ul.
2. Equilibrate column with TNE buffer and add labelling mix to top of column. Discard eluate.
3. Add 300ul of TNE to column and discard eluate.
4. Add 400ul of TNE to column and collect all eluate which contains labelled probe.
5. To measure activity, add 5ul of eluate to 10ml of Aquasol-2 solvent (NEN Research Systems) and count in scintillation counter. Probes with activity of >4 x 10⁸ cpm/ug DNA were used for hybridisation.
6. Rinse column with dilute Decon to reduce radioactivity prior to disposal.

2.9 Hybridisation of filters

2.9.1 Materials

50x Denhardt’s solution

5g Ficoll (Sigma)  
5g polyvinylpyrrolidene (Sigma)  
5g bovine serum albumin (Sigma)  
dH₂O to 500ml

Prehybridisation/hybridisation mix

6x SSC  
5x Denhardt’s solution  
0.5% SDS  
0.1% sodium pyrophosphate  
10mM EDTA  
10% dextran sulphate

Salmon sperm DNA

Dissolve 500mg of desiccated salmon sperm DNA (Sigma) in 50ml of distilled water, stirring to aid dissolution. Autoclave or sonicate to fragment DNA. Adjust concentration to 10mg/ml. Add 100ul/10ml per hybridisation.

Stringency washes

0.1% sodium pyrophosphate  
0.1% SDS  
0.1% - 2x SSC

2.9.2 Method

1. Both polythene bags and hybridisation bottles (Hybaid) can be used. Immerse filters briefly in boiling 0.1% SDS to remove residual agarose. Place directly into the polythene bags but soak in 2x SSC and roll in
mesh before putting into bottles. In both cases air bubbles should be carefully excluded.

2. Add 20 to 30 mls of prehybridisation solution and carefully heat seal bag or cap bottle.

3. Prehybridise for 2-6 hours at 65°C.

4. Boil labelled probe and 200-300ul of sonicated salmon sperm for 10 minutes and cool on ice for 5 minutes before adding to prehybridisation solution in bag or bottle.

5. Hybridise for 12-16 hours at 65°C.

6. Wash filters with 300ml to 500ml of stringency wash preheated to 65°C. Filters were removed from bottles for washing. Each was washed twice in 2x SSC,0.1% SDS and twice in 0.1x SSC,0.1% SDS.

7. Blot washed filter to remove excess wash fluid and wrap closely in Saran wrap.

8. In dark room, place in cassette with intensification screen and preflected Kodak X-OMat film applied to DNA side of filter.

9. Autoradiograph overnight at -70°C before developing. Subsequent exposure depends on signal intensity.

10. Strip filter by boiling for 5 minutes in 0.1% SDS.
2.10 Amplification of genomic DNA by the polymerase chain reaction

2.10.1 Materials

2x PCR buffer (for 10 PCR reactions)

100mM KCl  
20mM Tris PH 8  
3mM MgCl₂  
4mM each dATP, dCTP, dGTP, dTTP (Pharmacia)  
1μM each primer  
4μl Taq DNA polymerase (20 units) (Promega)  
dH₂O to 500μl

PCR Primers

Primer 1 - 5’-TTAGAGTTGCTTTACGTG-3’- was complementary to the negative strand segment 5’ of the major breakpoint region (Stetler-Stevenson et al, 1988). Primer 2 - 5’-GACTCCTTTACGTGCTGGTACCA-3’- corresponded to MC8 (Ngan et al, 1989) which lies within the minor cluster region. The third primer - 5’-ACCTGAGGACGGTGACCAGGGT-3’- was a JH consensus sequence complementary to the sequences at the 3' end of each (+)-strand J segment (Crescenzi et al, 1988). Initially, the JH primer was 5 bases shorter at the 3' end, but this resulted in multiple bands on amplification. Specificity was improved by the additional nucleotides.

2.10.2 Method

Every effort was made to reduce possible DNA contamination. Separate pipettes, tips and microfuge tubes were reserved for PCR reactions only. Gloves were worn throughout and changed frequently.
1. Make up enough 2x PCR buffer for 10 PCR reactions. Store at -20°C until required.

2. Thaw 2x PCR buffer, mix and pipette 50ul each into 500ul microfuge tubes. Discard any buffer not used.

3. Add volume of distilled water to 100ul, less volume required for 1-10ug of purified DNA.

4. Mix and overlay with 100ul of mineral oil (Sigma).

5. Pipette 1-10ug of purified genomic DNA into each tube. Open only one tube at any one time. Wipe tip of pipettor between samples with 100% ethanol and use new disposable tip for each sample.

6. Mix thoroughly and centrifuge briefly in Microfuge to separate oil and aqueous layers.

7. Amplify for 20-40 cycles in automatic thermal cycler (Perkin Elmer Cetus). Cycles for amplification of DNA from both major and minor breakpoint regions were as follows: denaturation, 94°C, 1 minute, annealing, 55°C, 2 minutes and synthesis, 72°C, 3 minutes.

To detect products, take 10ul from each reaction and separate by electrophoresis on a 2% agarose gel, stain with ethidium bromide and view under ultraviolet light. A 1Kb DNA ladder (Gibco BRL) was used for DNA size markers. For more specific identification of PCR sequences, make filters from gels by Southern blotting as described in Section 2.7, hybridise and wash as described in Sections 2.8 and 2.9.
Strong signals are usually apparent after 1-2 hours autoradiography.

To determine the detection limit of the PCR reaction, a sample with evidence of t(14;18) on Southern blotting was selected. The intensity of the rearranged band approached that of the germline fragment (Crescenzi, 1990). The sample was serially diluted with normal DNA in 10-fold dilutions and amplified as described above. After visualising the products on a gel, a band was detectable in the $10^{-4}$ dilution after 40 cycles.
CHAPTER 3

CYTOGENETIC ANALYSIS OF FOLLICULAR LYMPHOMA
3.1 Introduction

For several years, cytogenetic analysis of resected lymph nodes has been performed as part of the routine diagnostic work-up of patients with suspected non-Hodgkin’s lymphoma in Edinburgh. The presence of clonal karyotypic abnormalities has provided further evidence of malignancy. The complexity of the karyotypic changes which are found in non-Hodgkin’s lymphoma even at diagnosis make their analysis particularly challenging. The cytogenetic findings from seventy-two patients with B-cell follicular non-Hodgkin’s lymphoma are described here. Of particular interest is the incidence of t(14;18), which has been reported in almost 50% (Bloomfield et al, 1983) to 90% of cases of follicular lymphoma (Yunis et al, 1987) in the USA. The incidence of t(14;18) in European follicular lymphoma has been reported as 41%, although only molecular methods of detection were used (Pezzella et al, 1990a).

As well as t(14;18), the multitude of other cytogenetic abnormalities found in NHL are also discussed, as are sequential karyotypic changes in three patients and the cytogenetic findings in one patient with transformed follicular lymphoma.
3.2 Patient population and tumour characteristics

Cytogenetic analyses were attempted on malignant tissue removed from seventy-two patients who were found to have follicular non-Hodgkin's lymphoma. Forty patients were male. The age range was 26 to 83 years, with a median of 58 years. In 45 patients, the tissue was examined at the time of primary diagnosis and in 19 patients the tissue was studied at relapse after treatment with chemotherapy or radiotherapy. A further 2 patients had tissue examined at both diagnosis and relapse and one patient was analysed at two relapses. The time of analysis was unknown for 5 patients. Four patients had stage I disease at time of analysis, 10 patients were stage II, 19 had stage III disease and 25, stage IV. The stage of disease was unknown for 14 patients. Thirty eight patients remained alive at the time of the study with a median survival of 34 months, range 1 to 134 months. Twenty three patients had died, the median survival time from diagnosis being 32 months, range 5 to 143 months. Eleven patients had no survival data available.

All specimens were excised lymph nodes except case 30 (spleen), case 38 (bone marrow aspirate), case 46 (spinal mass) and case 71 (thyroid). The specimens were removed between October 1982 and November 1990 and the majority were reviewed histologically by a single pathologist, Dr A Krajewski, and classified according to the Working
Formulation (Rosenberg, 1982). All were B-cell lymphomas by conventional immunophenotyping. Twenty-six patients had follicular small cleaved cell lymphoma (Group B in the Working Formulation), 41 had mixed small and large cell disease (Group C) and 4 had follicular large cell lymphoma (Group D). One patient had transformed follicular lymphoma which had progressed from group C to high grade immunoblastic lymphoma.

3.3 Method

A piece of the same tumour mass which was studied histologically and immunologically was sent fresh for cytogenetic analysis. A small piece of this tissue was also frozen for later molecular analysis.

Cell suspensions were made and cultured according to methods described in Section 2.2. Where possible, both 24 hour and 48 hour synchronised cultures were set up and harvested as described previously. After slide making and staining, metaphases were found manually and analysed initially in some cases with the aid of photokaryotypes. However, analysis was subsequently improved by using a computer-based semi-automatic karyotyping system.

At least 20 metaphases were analysed per patient if possible. Karyotypic abnormalities were defined as clonal if identical structural abnormalities or additional chromosomes were present in two or more cells or if identical
chromosomes were missing from three or more cells. The presence of one normal cell defined a normal clone. Karyotypes were described according to the ISCN nomenclature (ISCN, 1981; 1985). Conventional 2 x 2 Chi-square analysis with Yate's correction was used to compare the frequency of karyotypic abnormalities between histologic sub-groups of follicular lymphoma.

3.4 Results

An abnormal karyotype was found in 65 patients (90.2%). Of these, three could not be fully described due to the poor quality of the metaphases and the complexity of the cytogenetic abnormalities. One patient yielded normal metaphases only and in six cases there were no divisions to analyse. The three patients studied twice yielded analysable abnormal metaphases on both occasions, making a total of 68 abnormal karyotypes for analysis. These are listed in full in Table 3.1. The following results consider those with abnormal karyotypes only. Where the incidence of a specific abnormality is considered, the patients analysed twice are counted only once.

3.4.1 Presence of normal metaphases

In addition to cells with clonal karyotypic abnormalities, normal metaphases were present in 37 (54%) of the 68 cases. Normal metaphases were present in 27/45 (60%) of those
Table 3.1  Karyotypes of 72 patients with follicular non-Hodgkin's lymphoma.  D/R determines the time of analysis at diagnosis or relapse.  NK=not known.

**Follicular small cell lymphoma - IWF group B**

<table>
<thead>
<tr>
<th>Case</th>
<th>Age/sex</th>
<th>D/R</th>
<th>Normal</th>
<th>Karyotype</th>
</tr>
</thead>
</table>
| 1.   | 55/M    | D   | 0      | 1) 46,XY,t(14;18) (7 cells)  
2) 47,as 1,+21,dup(1)(q25q44) (7 cells) |
| 2.   | 80/M    | D   | 0      | 1) 46,XY,t(14;18) (3 cells)  
2) 47,as 1,plus fragment (5 cell) |
| 3.   | 61/F    | R   | 0      | 1) 50,XX,+X,+2,+11,+13,+16,+21,t(14;18) (8 cells)  
2) 51,as 1, plus fragment (7 cells) |
| 4.   | NK/H    | D   | 27     | 1) 46,XY |
| 5.   | 62/M    | R   | 0      | 1) 52,XY,+X,+X,+3,+12,+12,+18,+21,del(3)(p13),t(14;18) (4 cells) |
| 6.   | 78/F    | R   | 0      | 1) 47,XX,t(18),del(6)(p21),t(14;18) (20 cells) |
| 7.   | 63/F    | R   | 2      | 1) 48,XX,+3,+8,der(3)(t(3;7)(q11;p13),del(10)(q22;25),t(14;18) (11 cells)  
2) 48,as 1, plus fragment - double minute (5 cells) |
| 8.   | 77/M    | D   | 0      | 1) 47,XY,+18,t(5;12)(q13;p13),t(14;18),t(16;17)(q22;25) (11 cells)  
2) 48,as 1, plus fragment (5 cells) |
| 9.   | 45/M    | D   | 0      | 1) 49,XY, + 3 tiny markers,t(1;12)(p13;q12),del(2)(q23;q27),  
del(3)(q13.2;q25.3),der(4)(t;4)(q33;p13),del(5)(q22q31),  
t(5;8)(14pter-q21;14q24-q32;8q24-qter;14pter-q24;14q21-qter),  
t(7;13)(p22;q14),del(8)(q13q22),del(12)(p11p13),t(14;18),i(17q)  
(20 cells) |
| 9b.  | 46/M    | R   | 0      | 1) as before,-9 (20 cells) |
| 10.  | 64/M    | R   | 2      | 1) 46,XY,t(14;18) (3 cells)  
2) 46,as 1,t(3;4)(q25;q21),del(10)(q22q24) (16 cells)  
3) 46,as 1,del(6)(q13q21),der(6)(q21)t(6;7),del(8)(p21),dup(15)(q15q21)  
(3 cells) |
| 11.  | 57/F    | D   | 3      | 1) 49,XX,+8,+10,t(14;18),+der(18) (4 cells)  
2) 46,XY,t(14;18),+der(18) (12 cells) |
| 12.  | 29/F    | D   | 7      | 1) 47,XX,der(6)(q13q27),t(14;18),+der(18) (12 cells) |
| 13.  | 65/M    | NK  | 3      | 1) 48,XY,-7,+12,+,2 markers,inv(2)(p13q36),t(5;22)(q13p11),  
t(8;14;14;18)(q24q22;q32q21),dup(12)(q13q24) (5 cells) |
<p>| 14.  | 61/M    | D   | 0      | 1) 46,XY,del(10)(q24.3),t(14;18),del(16)(q22) (12 cells) |</p>
<table>
<thead>
<tr>
<th>Case</th>
<th>Age/sex</th>
<th>D/R</th>
<th>Normal</th>
<th>Karyotype</th>
</tr>
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<tbody>
<tr>
<td>15.</td>
<td>NK/F</td>
<td>D</td>
<td>FAIL</td>
<td>1) 53,XX,+X,+7,+8,+9,+12,+18,+2markers,dup(2)(p13p21),del(4)(q15q25), t(14;18),dup(17)(q21q23),+der(18) (14 cells)</td>
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<tr>
<td>16.</td>
<td>73/M</td>
<td>D</td>
<td>FAIL</td>
<td>2) 49,XY,+Y,+8,+12,t(14;18),ins(17;10)(q11.2;q11.2q23.2) (10 cells)</td>
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<td>17.</td>
<td>43/F</td>
<td>R</td>
<td>19</td>
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<td>18.</td>
<td>30/M</td>
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<td>2) 50,as 1,+21 (16 cells)</td>
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<td>19.</td>
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<td>D</td>
<td>3</td>
<td>3) 51,as 2,+10 (2 cells)</td>
</tr>
<tr>
<td>20.</td>
<td>49/F</td>
<td>D</td>
<td>1</td>
<td>1) 46,XY,t(14;18) (11 cells)</td>
</tr>
<tr>
<td>21.</td>
<td>46/F</td>
<td>D</td>
<td>FAIL</td>
<td>2) 46,as 1,del(10)(q23.1q23.3) (6 cells)</td>
</tr>
<tr>
<td>22.</td>
<td>74/F</td>
<td>NK</td>
<td>FAIL</td>
<td>3) 48,XY,+21,12q+,t(14;18),+der(18) (22 cells)</td>
</tr>
<tr>
<td>23.</td>
<td>44/F</td>
<td>D</td>
<td>10</td>
<td>1) 47,XX,+X,t(14;18) (25 cells)</td>
</tr>
<tr>
<td>24.</td>
<td>56/M</td>
<td>D</td>
<td>0</td>
<td>1) 47,XX,+X,+Y,+8,del(2)(q13q23),del(14)(q15q25),+der(14)(q14;7)(q32;7) (4 cells)</td>
</tr>
<tr>
<td>25.</td>
<td>74/F</td>
<td>D</td>
<td>0</td>
<td>2) 47,as 1,del(22)t(del(2)q22)(q13;p13) (20 cells)</td>
</tr>
<tr>
<td>26.</td>
<td>44/F</td>
<td>D</td>
<td>5</td>
<td>3) 47,as 2,del(9)t(9;9)(p15q7) (7 cells)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>4) 49,XY,t(14;18),+der(18) (17 cells)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>5) 48,as 1,+8 (3 cells)</td>
</tr>
</tbody>
</table>

**Follicular mixed cell lymphoma - IWF group C**

<table>
<thead>
<tr>
<th>Case</th>
<th>Age/sex</th>
<th>D/R</th>
<th>Normal</th>
<th>Karyotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>27.</td>
<td>35/M</td>
<td>R</td>
<td>0</td>
<td>1) 54,XY,+X,+Y,+Y,+5,+10,der(11)t(11;11)(p34;q13),+der(2)t(2;12)(q35;q13),del(3)(p13),+del(3)(q15q25),+der(4)t(3;8) (p21;q22),+der(12)t(12;12)(q11;q24),t(14;18) (33 cells)</td>
</tr>
<tr>
<td>28.</td>
<td>37/M</td>
<td>D</td>
<td>17</td>
<td>2) 57,as 1,+3,+7,+8 (3 cells)</td>
</tr>
<tr>
<td>29.</td>
<td>63/M</td>
<td>R</td>
<td>0</td>
<td>1) 46,Y,-6,t(X;11)(p22;q32),+der(X),t(2;2)(q32;p25),+del(3)(p13),del(3)(p13),+del(5)(p13),del(7)(q26),+der(18)(q21q24),t(14;18) (9 cells)</td>
</tr>
</tbody>
</table>

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<table>
<thead>
<tr>
<th>Case</th>
<th>Age/sex</th>
<th>D/R</th>
<th>Normal</th>
<th>Karyotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>30.</td>
<td>40/F</td>
<td>D</td>
<td>0</td>
<td>1) 47,XX,+22,t(2;8)(q21;q22),del(6)(q15),t(14;18) (11 cells)</td>
</tr>
<tr>
<td>31.</td>
<td>72/F</td>
<td>D</td>
<td>0</td>
<td>1) 47,XX,+i(6p),ins(12;7)(q15;7),t(14;18) (19 cells)</td>
</tr>
<tr>
<td>32.</td>
<td>59/M</td>
<td>D</td>
<td>8</td>
<td>1) 45,XY,-12,-13,+marker,del(9)(q12),t(11;14)(q13;q32), der(15)t(15;7)(p13;7),der(17)t(13;17)(q22;p11) (20 cells)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2) 90,as 1,x 2 (3 cells)</td>
</tr>
<tr>
<td>33.</td>
<td>33/F</td>
<td>D</td>
<td>5</td>
<td>1) 85 = 2 x (46,XX,-17,+22,der(1)t(1;7)(p36;7),del(6)(q15), t(8;22)(q24;q11),del(13)(q12q14),t(14;18)),+X,+7,+9,-12,-15,-22 (14 cells)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2) 90,as 1,x 2 (3 cells)</td>
</tr>
<tr>
<td>34.</td>
<td>60/M</td>
<td>R</td>
<td>7</td>
<td>1) 48,XY,+21,t(14;18),+der(18) (3 cells)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2) 48,as 1,der(1)t(1;3)(p34;p13),del(12)(p11) (14 cells)</td>
</tr>
<tr>
<td>35.</td>
<td>43/M</td>
<td>R</td>
<td>0</td>
<td>1) 46,XY,t(14;18) (38 cells)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2) 46,as 1,del(1)(q42),der(6)(del(6;16)(q12;q27) (8 cells)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>3) 46,as 2,der(6),der(21)t(11;21)(q21;q22) (5 cells)</td>
</tr>
<tr>
<td>36.</td>
<td>39/F</td>
<td>D</td>
<td>0</td>
<td>1) 47,X,-X,+7,t(14;18),+der(18),+der(19)t(11;19)(q13.1;q13.3) (20 cells)</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>2) 48,as 1,+13 (4 cells)</td>
</tr>
<tr>
<td>37.</td>
<td>53/M</td>
<td>D</td>
<td>0</td>
<td>1) 48,XY,+21,t(14;18),+der(18) (3 cells)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2) 48,as 2,+22 (3 cells)</td>
</tr>
<tr>
<td>38.</td>
<td>70/F</td>
<td>D</td>
<td>4</td>
<td>1) 46,XX,del(7)(q22q32) (17 cells)</td>
</tr>
<tr>
<td>39.</td>
<td>70/M</td>
<td>D</td>
<td>0</td>
<td>1) 45,XY,+2,-4,-7,-15,+marker,del(1)(p21),t(6;16)(q13;p13), der(7)t(4;7) (q22p22),del(8)(q11),+del(9)(q11), der(13)t((13;7)(q24;2),t(14;18)(31 cells)</td>
</tr>
<tr>
<td>40.</td>
<td>64/M</td>
<td>D</td>
<td>8</td>
<td>1) 47,XY,+7,del(10)(q22q24),t(14;18) (26 cells)</td>
</tr>
<tr>
<td>41.</td>
<td>83/M</td>
<td>D</td>
<td></td>
<td>FAIL</td>
</tr>
<tr>
<td>42.</td>
<td>48/M</td>
<td>R</td>
<td>0</td>
<td>1) 48,XY,+18,dup(2)(p13p21),t(14;18),der(16)t(1;16)(q21.1;q12.1) (17 cells)</td>
</tr>
<tr>
<td>43.</td>
<td>67/M</td>
<td>R</td>
<td>0</td>
<td>NFA, but includes +X,+12,+inv(6q-),16p+,14q+,18q-</td>
</tr>
<tr>
<td>44.</td>
<td>52/M</td>
<td>D</td>
<td>3</td>
<td>1) 48,Xp,Y,+18,-22,+1p-,6q+,t(14;18),17p+,22q+ (10 cells)</td>
</tr>
<tr>
<td>45.</td>
<td>45/F</td>
<td>D</td>
<td>6</td>
<td>1) 46,XX,t(14;18) (95 cells)</td>
</tr>
<tr>
<td>46.</td>
<td>32/M</td>
<td>D</td>
<td>3</td>
<td>1) 47,XY,+7,der(7)t(7;7)(p22;q22),t(14;18) (20 cells)</td>
</tr>
<tr>
<td>Case</td>
<td>Age/sex</td>
<td>D/R</td>
<td>Normal</td>
<td>Karyotype</td>
</tr>
<tr>
<td>------</td>
<td>---------</td>
<td>-----</td>
<td>--------</td>
<td>-----------</td>
</tr>
<tr>
<td>47.</td>
<td>42/F</td>
<td>D</td>
<td>0</td>
<td>1) 47,XX,+8,dup(1)(q23q41),der(4)t(4;9?)(p16;q13) (29 cells) 2) 46,as 1,x 2 (3 cells)</td>
</tr>
<tr>
<td>48.</td>
<td>43/M</td>
<td>D</td>
<td>1</td>
<td>1) 46,XT,t(14;18),der(21)t(17;21)(q12?q121) (14 cells)</td>
</tr>
<tr>
<td>49.</td>
<td>54/M</td>
<td>D</td>
<td>3</td>
<td>1) 47,XT,+marker,t<a href="p36;q21;q13">1;13;5</a>,der(10)t(10q7?)(q25?q7), t(14;18) (7 cells)</td>
</tr>
<tr>
<td>50.</td>
<td>36/M</td>
<td>D</td>
<td>4</td>
<td>1) 46,XT,del(6)(p13q23?),t(14;18)(q32;q21) (13 cells)</td>
</tr>
<tr>
<td>51.</td>
<td>44/F</td>
<td>R</td>
<td>9</td>
<td>1) 47,XX,+7,del(10)(q22q24),t(14;18)(q32;q21) (30 cells)</td>
</tr>
<tr>
<td>52.</td>
<td>26/M</td>
<td>D</td>
<td>4</td>
<td>1) 47,XT,t(14;18)(q22q21),+der(18) (22 cells)</td>
</tr>
<tr>
<td>52b.</td>
<td>30/M</td>
<td>R</td>
<td>15</td>
<td>1) 46,XT,t(14;18)(q22q21) (5 cells) 2) 46,as 1,der(22)t(11;22)(q7;p11) (17 cells) 3) 46,as 1,der(4)t(1;4)(q21;p16) (3 cells)</td>
</tr>
<tr>
<td>53.</td>
<td>49/F</td>
<td>R</td>
<td>5</td>
<td>1) 48,XX,del(1)t(1;?) (p36?q?),del(2)(q13q33),+del(2), del(6)(q15q25),+del(6),der(12)t(12;7)(q24?) (3 cells) 2) 49,as 1,45 (3 cells) 3) 49,as 2,der(20)t(20;?) (17 cells)</td>
</tr>
<tr>
<td>54.</td>
<td>44/F</td>
<td>NK</td>
<td>2</td>
<td>1) 48,XX,+del(X)(q24) ,-18,t(14;18)(q32;q21),+der(18), +der(18) (7 cells) 2) 47,as 1,4 (4 cells)</td>
</tr>
<tr>
<td>55.</td>
<td>35/R</td>
<td>R</td>
<td>2</td>
<td>1) 46,XX,t(14;18)(q32q21) (3 cells) 2) 48,as 1,211,+der(18) (8 cells)</td>
</tr>
<tr>
<td>55b.</td>
<td>38/R</td>
<td>R</td>
<td>7</td>
<td>1) 49,as 2 above,4X,i(17q) (25 cells) 2) 49,as 1,del(3)(p2p2p4),del(5)(q14q23) (4 cells)</td>
</tr>
<tr>
<td>56.</td>
<td>68/F</td>
<td>R</td>
<td>6</td>
<td>1) 49,X,-X,+5,+7,+14,+ring,dup(1)(qter-q21;p36-qter), der(3)t(3;?) (q27?),del(4)(p13),i(6p),t(14;18)(q32?q21) (12 cells) 2) 50,as 1,-ring, 2 small markers (7 cells) 3) 51,as 2, but both Xs present (6 cells)</td>
</tr>
<tr>
<td>57.</td>
<td>32/F</td>
<td>R</td>
<td>0</td>
<td>1) 46,XX,der(1)t(1p7?),t(14;18)(q32?q21) (6 cells) 2) 47,as 1,i(6p) (2 cells) 3) 46,as 1,3q+,7q-,9p-,18,+der(18) (4 cells) 4) 46,as 1,17,18,-18,+der(18) (2 cells)</td>
</tr>
<tr>
<td>58.</td>
<td>52/M</td>
<td>D</td>
<td>0</td>
<td>1) 46,XY,t(14;18)(q32?q21) (3 cells)</td>
</tr>
<tr>
<td>59.</td>
<td>93/M</td>
<td>NK</td>
<td>2</td>
<td>1) 47,XY,+7,t(14;18)(q32?q21) (9 cells)</td>
</tr>
<tr>
<td>60.</td>
<td>58/M</td>
<td>D</td>
<td>8</td>
<td>1) 46,XY,t(14;18)(q32?q21) (3 cells) 2) 47,as 1,der(18) (19 cells)</td>
</tr>
<tr>
<td>Case</td>
<td>Age/sex</td>
<td>D/R</td>
<td>Normal</td>
<td>Karyotype</td>
</tr>
<tr>
<td>------</td>
<td>---------</td>
<td>-----</td>
<td>--------</td>
<td>-----------</td>
</tr>
<tr>
<td>61.</td>
<td>76/F</td>
<td>D</td>
<td>2</td>
<td>1) 49,XX,+2,+8,+18,t(3;14)(q27;q22) (16 cells)</td>
</tr>
<tr>
<td>62.</td>
<td>59/M</td>
<td>D</td>
<td>0</td>
<td>1) 46,XY,-4,-7,+ large marker, der(1)t(1;7)(q21;q22), der(8)t(8;q21), del(13)(q14q31), t(14;18)(q32;q21), der(18) (10 cells)</td>
</tr>
<tr>
<td>63.</td>
<td>62/F</td>
<td>D</td>
<td>0</td>
<td>1) 49,XX,+18,+21,t(12;?)p(25;?)t(14;18)(q32;q21), der(18) (9 cells)</td>
</tr>
<tr>
<td>64.</td>
<td>62/M</td>
<td>D</td>
<td>3</td>
<td>1) 46,XY,t(14;18)(q32;q21) (5 cells)</td>
</tr>
<tr>
<td>65.</td>
<td>83/F</td>
<td>NK</td>
<td>3</td>
<td>1) 56,XX,+2,+7,+8,+11,+13,+17,+19;t(1;7)(q42;q12), del(13)(p25), t(5;15)(q23;q22), dup(12)(q13.q21.2), t(14;18)(q13;q21), der(14) (16 cells)</td>
</tr>
<tr>
<td>66.</td>
<td>40/F</td>
<td>D</td>
<td>8</td>
<td>1) 47,XX,+8,del(18)(q22;q21), t(14;18)(q32;q21), del(15)(q13q15) (8 cells)</td>
</tr>
<tr>
<td>67.</td>
<td>32/F</td>
<td>R</td>
<td>0</td>
<td>1) 48,XX,+7,+21,t(1;21)(p36;q21) (5 cells)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2) 50,XX,+12,del(1)(q12) (4 cells)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>3) 51,XX,+12,del(1)(q12) (4 cells)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>4) 51,XX,+12,del(1)(q12) (4 cells)</td>
</tr>
</tbody>
</table>

**Follicular large cell lymphoma - IWF group D**

<table>
<thead>
<tr>
<th>Case</th>
<th>Age/sex</th>
<th>D/R</th>
<th>Normal</th>
<th>Karyotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>68.</td>
<td>55/M</td>
<td>R</td>
<td>FAIL</td>
<td>1) 56,XX,+2,+7,+18,del(13)(p11;pl1), t(1;7)(q42;q12), del(13)(p25), t(5;15)(q23;q22), dup(12)(q13.q21.2), t(14;18)(q13;q21), der(14) (16 cells)</td>
</tr>
</tbody>
</table>

**Follicular lymphoma - transformed**

<table>
<thead>
<tr>
<th>Case</th>
<th>Age/sex</th>
<th>D/R</th>
<th>Normal</th>
<th>Karyotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>69.</td>
<td>41/M</td>
<td>D</td>
<td>0</td>
<td>1) 56,XX,+3,+7,+18,del(13)(p11;pl1), t(8;14)(q24;q32) (25 cells)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2) 51,XX,+10,+13 (2 cells)</td>
</tr>
<tr>
<td>70.</td>
<td>79/F</td>
<td>D</td>
<td>1</td>
<td>1) 51,XX,-X,+2,-3,-4,-5,+7,+12,+15,+16,+17,-18,-18, -19,del(1)(p12p22), del(1)(q21q22), del(1)(q15q21), del(1)(t7;11)(q11;q25), del(1)(q13), t(14;18)(q32;q12), t(19;19)(q13;ql3) (15 cells)</td>
</tr>
<tr>
<td>71.</td>
<td>72/F</td>
<td>D</td>
<td>0</td>
<td>1) 46,XX,t(3;9)(q22;q22), der(12)t(3;12)(p21;q24) (10 cells)</td>
</tr>
</tbody>
</table>

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studied at diagnosis, prior to treatment, and in 10/23 (43%) studied at relapse but the difference was not statistically significant (p<0.5).

3.4.2 Modal number of chromosomes and number of clones

The modal number of chromosomes in the predominant clone was 46 or 47. The majority of patients (90.9%) examined at diagnosis had one or two clones whereas 38% of those examined at relapse had three or more clones present (p<0.025). In most cases, the abnormalities in the major and minor subclones were clearly related (Table 3.1). Only three patients had a single clonal abnormality – the reciprocal translocation t(14;18)(q32;q21) in case 45 and 58 and del(7)(q22q32) in case 38. The remainder of karyotypes were complex and contained multiple clonal defects.

The three patients who were studied on two occasions showed clonal evolution with acquisition of further cytogenetic abnormalities. In all three cases, t(14;18) was present in the initial specimen and was retained on subsequent analysis. Case 52 lost the extra der(18) chromosome present at diagnosis but developed two further subclones with abnormalities of the long arm of chromosome 1. In case 55, the karyotype at relapse had gained an X chromosome and had acquired i(17q) and deletions of 3p and 5q. Case 9 had a very complex karyotype at diagnosis with the only change at relapse being the loss
of chromosome 9. Despite the development of further karyotypic changes, only case 9 demonstrated histological progression - from follicular small cleaved cell to mixed cell lymphoma.

3.4.3 Incidence of t(14;18)(q32;q21)

The reciprocal translocation t(14;18)(q32;q21) (Figure 3.1) was present in 53 of 65 patients (81%) (Table 3.2) and was significantly associated with follicular small and mixed cell disease (p<0.005). It was not present in the three patients with follicular large cell lymphoma. The translocation occurred as the sole abnormality in only two cases but in several instances was associated with a single other abnormality such as +21 or +18. It also occurred as the single clonal abnormality of a major clone in association with subclones which had acquired further cytogenetic changes. Case 33, with follicular mixed cell lymphoma, had both t(8;22)(q24;q11) and t(14;18) and case 72, the transformed follicular lymphoma, had both t(14;18) and t(8;14)(q24;q32).

3.4.4 Other 14q32 abnormalities

Only two patients with follicular small cleaved cell lymphoma lacked t(14;18) and both had other abnormalities of 14q32. One patient had the complex translocation t(8;14;14;18)(q24;q22;q32;q21) and another had a translocation
Figure 3.1 Digitalised karyotype of Case 45, showing 46,XX,t(14;18). The translocated chromosomes are arrowed.
Table 3.2  Incidence of t(14;18)(q32;q24) in histologic subgroups of follicular lymphoma.

<table>
<thead>
<tr>
<th>histology</th>
<th>t(14;18)+ve</th>
</tr>
</thead>
<tbody>
<tr>
<td>small cell</td>
<td>19/21 (90%)</td>
</tr>
<tr>
<td>mixed cell</td>
<td>33/40 (82%)</td>
</tr>
<tr>
<td>large cell</td>
<td>0/3 (0%)</td>
</tr>
<tr>
<td>transformed</td>
<td>1/1 (100%)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>53/65 (81%)</strong></td>
</tr>
</tbody>
</table>
involving 14q32 and an unknown donor chromosome. In group C, one patient had t(11;14)(q13;q32) and another t(3;14)(q27;q32). One patient with a partially analysable karyotype had a 14q+ chromosome of unknown origin. Four patients had no visible abnormalities of chromosome 14. Of the three patients with follicular large cell lymphoma, one had t(8;14)(q24;q32) and the other two had no visible abnormalities of chromosome 14.

3.4.5 Karyotypic abnormalities other than 14q32

The karyotypes of most patients contained three or more cytogenetic abnormalities. The most common numerical abnormality was trisomy 7 which occurred in 18 cases (27%). An additional der(18)t(14;18) chromosome was observed in 16 cases (24%) and trisomy 18 in 11 (16%). Two of these cases had both trisomy 18 and +der(18). One case had lost its normal 18 but had two copies of der(18). Brief survival details for those patients with +der(18) are given in Table 3.3. Twelve of these patients were analysed at diagnosis.

Three other numerical abnormalities were observed in at least 15% of patients – an additional X chromosome in 12 (18%), trisomy 21 in 11 (16%) and trisomy 8 in 10 (15%).

Thirteen patients (20%) had deletions involving 6q with breakpoints ranging from q12 to q27. Deletions of 10q were present in 7 cases (11%) with the majority being interstitial
Table 3.3 Survival of patients with +der(18), from time of diagnosis and time of karyotyping. A + sign indicates that the patient is still alive. m = months.

<table>
<thead>
<tr>
<th>Case number</th>
<th>Survival from diagnosis</th>
<th>Survival from karyotyping</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>78m</td>
<td>78m</td>
</tr>
<tr>
<td>11</td>
<td>51m+</td>
<td>51m+</td>
</tr>
<tr>
<td>12</td>
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- del(10)(q22q24).

There were multiple other translocations, deletions and numerical abnormalities which occurred less frequently and which had more diverse breakpoints. These included trisomy 12, deletions of 3p and abnormalities of both arms of chromosome 1. No deletions involving 13q32 were observed but two patients had deletions with breakpoints at 13q12-q14. Four patients had trisomy 2 and 4 had other abnormalities of 2p, resulting in dup(2p). Four of these patients remained alive 10 to 65 months after karyotypic analysis and two had died, one 5 months following diagnosis from squamous lung carcinoma and the second 22 months from diagnosis of lymphoma. In the remaining two patients, survival details were unknown.

Apart from +der(18) which was only observed in patients with t(14;18), the abnormalities described were found in both t(14;18) positive and t((14;18) negative cases, and were present at diagnosis and after treatment. The only karyotypic abnormality which was associated significantly with a histologic subgroup of follicular lymphoma was t(14;18).

3.5 Discussion

This description of 68 complex karyotypes from 65 patients with follicular lymphoma represents several years of skilled preparation and analysis. The success rate in
obtaining an abnormal karyotype was approximately 90%, which compares with the best of the published series (Yunis et al, 1987). Failure to obtain abnormal metaphases was probably due to failure of the malignant cells to divide in culture, rather than inadequate numbers of malignant cells as all biopsies were performed at time of active disease.

The finding of multiple clones at diagnosis and more especially at relapse is in contrast to other workers (Bloomfield et al, 1983; Kaneko et al, 1983; Yunis et al, 1987) where a single clone was most frequently seen. This may reflect the quality of the preparations and the diligence of the cytogeneticist, rather than the nature of the lymphoma (Ross et al, 1989).

The significant presence of three or more clones in those analysed at relapse demonstrates the increased complexity of karyotypic abnormalities as lymphoma progresses. The majority of sub-clones were clearly related to the parent clone. Other workers have also shown that karyotypic abnormalities become more complex after treatment (Offit et al, 1991a) although the type of karyotypic abnormality remains much the same (Koduru et al, 1987; Juneja et al, 1990). Unlike chronic myeloid leukaemia, where the appearance of secondary cytogenetic abnormalities heralds the onset of more aggressive disease (Singh et al, 1986), the acquisition of further cytogenetic defects in non-Hodgkin’s lymphoma is not always
associated with histologic progression (Sanger et al, 1987; Levine et al, 1990) as confirmed here by two of the three patients studied twice.

The association of the reciprocal translocation t(14;18)(q32;q21) with follicular lymphoma was confirmed in this series by the finding of the translocation in 81% of those with abnormal karyotypes; 90% of those with follicular small cell disease had the translocation. Several groups have also reported a particular association with follicular small cell disease (Levine et al, 1985; Koduru et al, 1987; Juneja et al, 1990) and in one series t(14;18) was found in 100% of cases (Yunis et al, 1987). The crucial importance of t(14;18) in the development of follicular lymphoma has been suggested by the presence of the translocation as the sole visible cytogenetic abnormality in several patients (Yunis et al, 1987). However, just two patients in this series had t(14;18) as the only cytogenetic abnormality, the remainder having more complex karyotypes.

The frequent involvement of 14q32 in karyotypic abnormalities in those with non-Hodgkin's lymphoma (Bloomfield et al, 1983) was confirmed by this series where 100% of those with follicular small cell disease had cytogenetic lesions at this locus, with 90% of these being t(14;18). All but 10% of those with mixed cell disease had 14q32 abnormalities, as did 33% of those with large cell disease.
The abnormalities of 14q32 other than t(14;18) were variable. The reciprocal translocation t(8;14)(q24;q32), found in one patient with follicular large cell lymphoma, is usually associated with high grade Burkitt’s and non-Burkitt’s lymphoma but has also been reported in low grade disease (Fifth Workshop, 1987; Koduru et al, 1987; Juneja et al, 1990). Both t(14;18) and t(8;14) were observed in the one patient with transformed follicular lymphoma, analysed only after histological progression. Sham et al (1989) showed that progression of follicular large cell lymphoma with t(14;18) to Burkitt’s lymphoma was associated with acquisition of t(8;14)(q24;q32). A similar event may have occurred in this case, paralleling the in vivo findings in mice, where bcl-2 and c-myc cooperate to produce aggressive lymphoid tumours (Strasser et al, 1990). More rarely, the variant translocation of Burkitt’s lymphoma, t(8;22)(q24;q11), has also been described in follicular lymphoma (Juneja et al, 1990) and was seen here in one patient.

One patient with follicular mixed cell lymphoma had t(11;14)(q13;q32), which is more commonly associated with chronic lymphocytic leukaemia or diffuse small cell (Levine et al, 1985) or diffuse large cell lymphoma (Levine et al, 1985; Koduru et al, 1987; Schouten et al, 1990). However, the translocation has previously been described in follicular mixed cell lymphoma (Schouten et al, 1990).
Trisomy 18 or +der(18)t(14;18) occurred in 9 and 14 cases respectively. A further two cases had both +18 and +der(18). This incidence of +der(18) is much higher than previously reported. Yunis et al (1987) reported 11/71 patients with follicular lymphoma as having +18 or dup(18q) but no instances of +der(18). Speaks et al (1987) described +der(18) in a subclone of one patient with follicular mixed cell lymphoma. An additional der(18) was also found in 2/33 patients with large cell lymphoma (Bloomfield et al, 1983). In a large series encompassing all subgroups of lymphoma, an extra der(18) was observed in 6/57 cases with t(14;18) (Fifth Workshop, 1987).

In contrast, Fukuhara et al (1988) described +der(18) in 6/10 Japanese patients with t(14;18) and other complex karyotypic abnormalities. All had a history of follicular lymphoma or diffuse large cell lymphoma. Five were studied at diagnosis and one after relapse and intensive chemotherapy. The majority had extra-nodal disease and a poor prognosis. These workers interpreted the presence of an extra der(18) as the equivalent of the appearance of an additional Philadelphia chromosome in chronic myeloid leukaemia, when an accelerated phase of disease is then expected.

However, in this series, +der(18) was found in 12/16 patients at diagnosis, with 9 remaining alive 2 to 74 months
after karyotypic analysis. Two patients with +der(18) found in relapsed karyotypes are alive 1 and 10 months after analysis. Two patients died 48 and 42 months after +der(18) was identified. This suggests that, unlike Japanese lymphoma, +der(18) is commonly found in diagnostic karyotypes in European lymphoma and is not associated with disease transformation or a particularly poor prognosis. It is worth noting that in poorly banded preparations it is difficult to distinguish der(18) from its normal counterpart. Also, der(18) can be lost during karyotypic evolution, as illustrated by case 52. Its loss in cell culture has also been described, as has its absence in karyotypes with 14q+ of unknown origin and molecular evidence of t(14;18) (Lipford et al, 1987). These factors may explain in part the difference in incidence of +der(18).

The majority of other numerical abnormalities described here are well-recognised karyotypic findings in both follicular and diffuse non-Hodgkin's lymphomas. Trisomy 7, found in over 25% of cases, has been frequently described in follicular lymphoma (Bloomfield et al, 1983; Levine et al, 1985; Koduru et al, 1987; Speaks et al, 1987; Yunis et al, 1987; Offit et al, 1991b). No significant association of trisomy 7 with a particular subgroup of follicular lymphoma was observed here and other workers have similarly found no histologic association (Juneja et al, 1990). However, +7 has
been associated with follicular large cell disease (Levine et al, 1985; Koduru et al, 1987) or follicular mixed and large cell disease (Yunis et al, 1987).

The presence of an additional X chromosome in 14 patients is also not unusual as gains of this chromosome are particularly common in non-Hodgkin's lymphoma, occurring in all histologic subgroups (Juneja et al, 1990). Trisomy 8 is a fairly frequent finding in follicular NHL (Yunis et al, 1987; Schouten et al, 1990) and occurred in 11% of cases here, distributed throughout all follicular histologic subtypes.

This study did not confirm the association of trisomy 8 with mixed cell disease (Koduru et al, 1987) or mixed and large cell disease (Levine et al, 1985). Both trisomy 21 and trisomy 12 are also relatively frequent in NHL (Yunis et al, 1987; Offit et al, 1991b). As in some series (Juneja et al, 1990; Schouten et al, 1990), no specific association with a follicular subtype of lymphoma was observed here, although +21 has been associated with follicular large cell disease (Yunis et al, 1987) and +12 with follicular mixed and large cell lymphoma (Yunis et al, 1987).

Almost all the other karyotypic abnormalities described in this series have been reported previously. No recurring structural defects were associated with a particular histologic subtype. Deletions of 6q are especially common in non-Hodgkin's lymphoma and occur in all histologic
subgroups (Rowley et al, 1980; Yunis et al, 1984a). Yunis et al (1987) found del(6q) particularly in association with follicular mixed or large cell disease. Levine et al (1985) found no significant association between abnormalities of chromosome 6 and follicular lymphoma in their large series encompassing all histologic subgroups of NHL.

Seven patients had deletions of 10q, the majority being interstitial deletions involving 10q22q24. Yunis et al (1987) did not report del(10q) as a frequent finding in their series of 71 patients with follicular lymphoma. Speaks et al (1987), however, described three cases of del(10q)(q23-25) in follicular mixed cell lymphoma. Juneja et al (1990) reported 11/147 cases of non-Hodgkin’s lymphoma with deletions at 10q22. The 5th Workshop (1987) described 5 patients with breaks at 10q22 and 5 with breaks at 10q24. Offit et al (1991b) found breaks at 10q22-24 in 7% of patients with low grade histology. Bloomfield et al (1983) had infrequent abnormalities of 10q but trisomy 10 was described in 7 cases, occurring in both follicular and diffuse large cell lymphoma.

Few studies have reported sequential karyotypes in non-Hodgkin’s lymphoma (Sanger et al, 1987; Yunis et al, 1987; Armitage et al, 1988; Levine et al, 1990) but acquisition of secondary karyotypic abnormalities in association with disease progression has been well described in other
haematological malignancies (Nowell, 1986). In low grade lymphoma, several cytogenetic abnormalities have been associated with advancing lymphoma. These include abnormalities of chromosomes 6 and 7 (Richardson et al, 1987), 2, 3, 6, 7, 12 and 18 (Yunis et al, 1987), abnormalities of chromosome 1 (Sanger et al, 1987; Levine et al, 1990) and further rearrangements at 14q32 (Levine et al, 1990). Offit et al (1991a), in a large series of 434 cases of non-Hodgkin’s lymphoma, found an increased number of breakpoints and marker chromosomes in those patients analysed after treatment. However, the distribution of the most frequent translocations and numerical and structural aberrations was similar between the pre- and post-treatment groups.

In this series, three patients were analysed on two occasions. The retention of t(14;18) in sequential karyotypes as seen in all three cases is well-recognised (Sanger et al, 1987; Yunis et al, 1987; Levine et al, 1990). Secondary abnormalities in these three cases included abnormalities of 1q, deletions of 3p and the development of isochromosome 17q (i(17q)). The development of i(17q) is a frequent secondary event in chronic myeloid leukaemia, heralding the onset of the accelerated phase of the disease (Singh et al, 1986). The loss of all or part of 17p has been described as a probable secondary abnormality in diagnostic karyotypes (Yunis et al, 1987) or as a novel karyotypic change
on sequential analysis (Levine et al, 1990) and in both situations is associated with a poor prognosis.

3.7 Conclusions

The karyotypes described in this thesis were derived from a group of 72 patients who, although all having follicular NHL, varied greatly in histologic subtype, age, stage and time of analysis. Complete survival statistics were not available for 15% and almost 50% remained alive at the time of writing. It is worth remembering that the median survival of patients with follicular lymphoma, irrespective of treatment, is 4 to 10 years (Gallagher et al, 1986; Horning et al, 1984). The histological subtype remains the most important factor in determining survival — those with follicular small and mixed cell disease have a better prognosis than follicular large cell lymphoma (Rudders et al, 1979) and follicular disease has a much better outlook than diffuse lymphoma (Anderson et al, 1982).

The link between cytogenetic abnormalities present at diagnosis and prognosis in NHL is not so clear-cut as it is in other hematologic malignancies. Similarly, the finding of additional karyotypic abnormalities on sequential analysis of lymphomas is not as predictive of disease progression as in chronic myeloid leukaemia. In the large series discussed here, the karyotypic features in follicular NHL
which are considered to be of possible prognostic significance (the presence of t(14;18) as a single abnormality, trisomy 2 or dup(2p) and trisomy 3 or dup(3p)) were found in too few patients to permit statistical analysis. However, the karyotypic abnormalities described are very similar to those of many other large series which considered the possible prognostic significance of cytogenetics (Offit et al, 1991b; Schouten et al, 1990; Kaneko et al, 1983; Levine et al, 1988; Kristoffersson et al, 1987; Yunis et al, 1987). Their conflicting findings were fully discussed in Chapter 1.

The contribution of cytogenetics to the understanding of the pathogenesis of non-Hodgkin’s lymphoma is not in doubt. The finding of recurrent, non-random chromosomal aberrations in over 90% of patients with follicular lymphoma described here confirms the link between genetic abnormalities and malignant disease. The identification of oncogenes such as c-myc and bcl-2 residing at breakpoint sites of recurrent translocations has helped to unravel many of the factors controlling normal and neoplastic cell growth and differentiation.

Despite these advances, for the individual patient with follicular non-Hodgkin’s lymphoma cytogenetic analysis does not yet play a crucial role in the diagnosis and management of his disease. The karyotypic abnormalities discussed are not unique to follicular disease but are found throughout all
histologic subgroups of non-Hodgkin's lymphoma, including T-cell disease, and also in other malignant tumours (Heim et al, 1987). The complexity of the karyotypes, even at diagnosis, makes it difficult to ascertain the genetic lesions of primary importance. Although cytogenetics can demonstrate the clonal origin of lymphoid tumours, other techniques such as immunophenotyping may be more rapid and specific.

The results of karyotypic analyses must always be interpreted with caution. The most insurmountable problem of cytogenetics is that a karyotypic abnormality, in order to be visible by standard cytogenetic techniques, requires the rearrangement or deletion of 10⁷ base pairs of DNA. It has been shown using molecular techniques that highly significant genetic rearrangements occur without visible cytogenetic abnormalities in chronic myeloid leukaemia (Bartram et al, 1986) and other workers have reported submicroscopic rearrangements of chromosomes 14 and 18 in follicular lymphoma (Lipford et al, 1987).

In addition, cytogenetics is too insensitive to detect minimal disease and its requirement for viable dividing cells makes it an unsuitable technique for the investigation of stored or fixed material. Despite these drawbacks, cytogenetic analysis of non-Hodgkin's lymphoma continues to contribute to our understanding of malignancy.
(Levine et al, 1989). The identification of oncogenes at translocation breakpoints in both B and T-cell lymphoma and the recognition of the importance of the immunoglobulin and T-cell receptor gene loci in the development of lymphoid malignancy are major breakthroughs in the comprehension of cancer. Who yet knows what important genes may be involved in the multitude of recurrent but unmapped translocations, deletions and duplications in non-Hodgkin’s lymphoma?
CHAPTER 4

MOLECULAR DETECTION OF $t(14;18)$ IN FOLLICULAR LYMPHOMA
MOLECULAR DETECTION OF t(14;18) IN FOLLICULAR LYMPHOMA

4.1 Introduction

The precise incidence of the reciprocal translocation t(14;18) in follicular lymphoma has not yet been established. Cytogenetically, t(14;18) has been demonstrated in 55% (Bloomfield et al, 1983) to 85% (Yunis et al, 1987) of patients with follicular lymphoma. Using Southern blotting, Weiss et al (1987) detected bcl-2 rearrangements compatible with t(14;18) in almost 90% of American patients but the molecular incidence in European follicular lymphoma has been reported as only 41% (Pezzella et al, 1990a). The detection of t(14;18) is clinically important as the presence of the translocation may be of prognostic significance, especially in large cell lymphoma (Yunis et al, 1987; 1989).

Failed karyotypic analyses, possible sub-microscopic bcl-2 rearrangements (Lee et al, 1987b) and the failure of Southern blotting to detect all cytogenetically evident t(14;18) (Weiss et al, 1987) suggest that a combination of techniques are required to more accurately determine the frequency of t(14;18) in diagnostic specimens. The presence of a detectable molecular rearrangement of bcl-2 provides a stable clonal marker of disease (Crescenzi et al, 1988). In the series of patients described here, both Southern blotting and PCR were used to detect t(14;18) in 49 patients with follicular lymphoma, the majority of whom had been
successfully karyotyped. Cytogenetic results were described in Chapter 3. This forms one of the largest series of follicular lymphoma studied by all three techniques.

4.2 Patient population and tumour characteristics

Cytogenetic details of patients analysed molecularly have been given in Section 3.4. Cases 1 to 9, 27 to 38, 70 and 71 were excluded from molecular analysis as no stored tumour tissue was available. A further five cases were excluded from Southern blotting but analysed by PCR - only degraded DNA was obtained from cases 22 and 54, and 3 cases (55,56 and 72) had insufficient material for Southern blotting. Of the six patients who had unsuccessful cytogenetic analysis, five were analysed molecularly. Of the three patients studied twice by cytogenetics, only two had stored material for molecular analysis, from the second specimen only. A further two patients had both diagnostic and relapsed nodes available for molecular analysis - cases 40 and 49. The relapsed nodes had not been submitted for cytogenetics.

Therefore, 44 patients were analysed by both molecular techniques and 5 by PCR alone. The 49 patients comprised 17 with follicular small cell lymphoma, 29 with mixed small and large cell disease, 2 with large cell lymphoma and one with transformed follicular lymphoma. Genomic DNA was also
extracted from placental tissue, an additional 20 non-
Hodgkin's lymphomas of non-follicular origin with no
cytogenetic evidence of t(14;18) and from one reactive node
and two nodes infiltrated with metastatic tumour. These
samples were also analysed molecularly by both techniques,
and served as a control population.

4.3 Method

4.3.1 Southern blotting

Purified genomic DNA was extracted as described in
Section 2.3 from either frozen chunks of lymph node or thick
sections cut from stored frozen section blocks. After
digestion with restriction enzymes (BclI, BamHI, EcoRI or
HindIII), the DNA was fractionated according to size by
electrophoresis on 0.8% agarose gels and transferred to
Hybond-N nylon membranes by the method of Southern (1975).
The membranes were then air-dried and baked, prehybridised,
hybridised and washed as described in Section 2.9. The
molecular probes used were described fully in Section 2.8 and
were radioactively labelled by the hexamer primer method
(Feinberg et al, 1983). The bcl-2 probe detected rearrangements
of bcl-2 at the major breakpoint region (mbr) (Tsujimoto et
al, 1985b) and the pfl-2 probe at the minor cluster region
(mcr) (Cleary et al, 1986b).
4.3.2 Polymerase chain reaction

Approximately 1ug to 10ug of purified genomic DNA was used per amplification. Primers for the major and minor breakpoint regions of bcl-2 and the consensus primer for the JH region of the immunoglobulin heavy chain gene were described in Section 2.10.1. DNA amplification was performed as outlined in Section 2.10.2, using an Automatic Thermal Cycler (Perkins Elmer Cetus). PCR products were visualised by ethidium bromide staining of 2% agarose gels. Filters were made from selected gels and hybridised with mbr and mcr probes as described in Sections 2.6 to 2.9. Autoradiography was carried out for only 1 to 2 hours because of the intensity of the signal obtained.

4.4 Results

The results of both Southern blotting and PCR analysis are summarised in Table 4.1, which also includes brief cytogenetic details.

4.4.1 Southern blotting

44 patients with follicular lymphoma were analysed by Southern blotting. Clonal rearrangements of the immunoglobulin heavy chain locus were detected in thirty eight of thirty nine patients probed with the JH probe, confirming the presence of a sizeable population of clonal B-cells. One patient
(case 15), in whom cytogenetic analysis had failed, showed a germline band only. Thirty patients showed rearrangement of bcl-2 (68%) (figures 4.1 and 4.2). Twenty nine of these patients had karyotypic evidence of t(14;18).

Conventional electrophoresis therefore detected t(14;18) in 29/32 (90%) patients known to have the translocation cytogenetically. Seventy per cent of breakpoints fell within the major breakpoint region, the remainder being detectable by the pFL-2 probe. The majority of those with breakpoints within the major region showed a comigrating fragment on reprobing with JH, confirming juxtaposition of chromosome 14 and chromosome 18 DNA. Only three of nine patients with mcr breakpoints had comigrating JH fragments. Additionally, bcl-2 rearrangements were detected by only one restriction enzyme in five of the nine. All had t(14;18) detectable cytogenetically.

No rearrangement of bcl-2 was detected in cases with 14q+ chromosomes of unknown origin or in case 13, which had the variant translocation t(8;14;14;18). However, one patient, case 67, with clonal cytogenetic abnormalities other than t(14;18), had a rearranged band after digestion of DNA with Bcl1 enzyme only and probing with the mbr probe. Further analysis after digestion with BamH1, EcoR1, HindIII and PstI showed no rearranged bands. There was no comigration of the rearranged fragment with a JH band.

Page 103
Table 4.1 Cytogenetic and molecular detection of t(14;18)(q32;q21). Cytogenetic evidence of t(14;18) is compared with its detection by Southern blotting and PCR. The pattern of rearrangement after digestion with several restriction enzymes is shown. JH column lists banding pattern after reprobing of filters with immunoglobulin heavy chain probe. R=rearranged; G=germline; C=comigrating band; ND=not done.

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continued on page 105
Table 4.1  Cytogenetic and molecular detection of t(14;18)(q32;q21). Cytogenetic evidence of t(14;18) is compared with its detection by Southern blotting and PCR. The pattern of rearrangement after digestion with several restriction enzymes is shown. JH column lists banding pattern after reprobing of filters with immunoglobulin heavy chain probe. R=rearranged; G=germline; C=comigrating band; ND=not done.

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Figure 4.1  Detection of bcl-2 rearrangements at the major breakpoint region by Southern blotting. All lanes contain DNA extracted from follicular lymphomas, digested with EcoRI restriction enzyme. Hybridisation of the same filter sequentially with bcl-2 and JH probes. G=Germline bands. Comigrating bands are arrowed, confirming juxtaposition of chromosome 14 and 18 DNA. Lane 1 shows no rearrangement at the mbr. Compare with Figure 4.2
Figure 4.2  The same filter as in Figure 4.1, but rehybridised with pfl-2, the probe to the minor breakpoint cluster region. Lane 1 shows bcl-2 rearrangement at the mcr, and the rearranged band of approximately 18kb comigrates with the JH fragment in lane 1, Figure 4.1. G=Germline bands.
Of the five patients with failed cytogenetic analysis, four were analysed by Southern blotting and only one showed evidence of bcl-2 rearrangement.

The two patients studied at both diagnosis and relapse had identical molecular rearrangements on subsequent analysis of the relapsed nodes, confirming the stability of the translocation as a molecular marker.

Clonal JH rearrangements were detected in all control lymphomas but no bcl-2 rearrangements were detected in any of the control DNA samples.

4.4.2 Polymerase chain reaction

Forty nine patients were analysed by PCR. Twenty four patients (49%) had a detectable band after amplification of genomic DNA (figure 4.3). Those with breakpoints falling at the major breakpoint region had bands of around 250bp to 400bp in size, whereas those amplified by primers from the minor cluster region had bands of approximately 900bp. Specific PCR bands were seen only in those with cytogenetic or Southern blotting evidence of t(14;18) and in one patient (case 22) who had failed cytogenetics and sufficient DNA for PCR only.

PCR amplification yielded a single detectable band in 22/36 (61%) patients with cytogenetic evidence of t(14;18).
Figure 4.3  Detection of t(14;18) by the polymerase chain reaction.  PCR products have been electrophoresed through a 2% agarose gel and stained with ethidium bromide.  Markers are in base pairs.  Lane 1 contains no DNA, lane 2 DNA from a reactive node only.  Lanes 3,4,6,7 and 8 contain DNA from follicular lymphomas with cytogenetic evidence of t(14;18).  Amplification with primers from the mbr has produced a specific positive band in each lane.  Lane 5 also contains DNA from a t(14;18) containing lymphoma and the larger band was produced after amplification using mcr primers.  The indistinct bands of approximately 120bp seen in several lanes were non-specific and their intensity often paralleled the amount of DNA added per reaction.
PCR was positive in 18/20 (90%) of those with breakpoints at the mbr on Southern blotting but in only 2/9 (22%) of those with mcr breakpoints. Identically sized bands were obtained on each occasion from the two nodes analysed at diagnosis and subsequent relapse. Specificity of positive bands was confirmed by hybridisation of selected filters with the appropriate probe.

Amplification of DNA from lymphomas without t(14;18) frequently resulted in a pattern of multiple non-specific bands or no detectable PCR product. Tubes with no DNA, placental DNA or DNA from reactive nodes were persistently negative.

4.5 Discussion

This simultaneous cytogenetic and molecular study of 49 patients with follicular lymphoma comprises one of the largest series from a single centre. Cytogenetic analysis proved the most reliable method of identifying the translocation, detecting t(14;18) in 36/49 (73%) of cases. Southern blotting demonstrated t(14;18) in 30/44 (68%) and PCR in 24/49 (48%). The pattern of detection is similar to that of Horsman et al (1990) who described simultaneous cytogenetic and molecular analysis of 37 patients with follicular lymphoma. Thirty six had karyotypic evidence of t(14;18).
Southern blotting detected bcl-2 rearrangements in 30/37 (81%) and PCR was positive in 78%.

In contrast, Chaganti et al (1989) found that the use of molecular probes to detect t(14;18) substantially increased the incidence of the translocation. Of 19 patients with follicular lymphoma, 9 had cytogenetic evidence of t(14;18) but 17 had molecular rearrangements of bcl-2. However, their cytogenetic failure rate approached 50% and undoubtedly the cytogenetic incidence of t(14;18) would have been much higher with more satisfactory preparations.

There is no evidence that karyotypic success is influenced by the presence of t(14;18) as there was no significant difference in the incidence of the translocation as detected by PCR in those with failed or successful cytogenetic analysis. Only two additional cases were detected molecularly, giving an overall incidence of t(14;18) of 38/49 (77%).

Failure to demonstrate cytogenetically visible t(14;18) by Southern blotting, as occurred in three cases, is probably due to the breakpoints being undetectable by the combination of restriction enzymes and probes used (Weiss et al, 1987) and is one of the major disadvantages of relying solely on molecular methods for detection of the translocation. It is unlikely that failure to detect t(14;18) by
Southern blotting was due to insufficient translocation-bearing cells being present as specimens were from diagnostic tissue and t(14;18) was detectable by less sensitive cytogenetic techniques.

It is conceivable that use of the 5' probe (Tsujimoto et al, 1987) may have increased the incidence of molecular detection of t(14;18) although the number of breakpoints falling within this region is very small (Weiss et al, 1987). Other workers have similarly failed to demonstrate cytogenetically visible t(14;18). Lee et al (1987b) failed to detect t(14;18) by Southern blotting in 3 of 10 patients known to have the translocation cytogenetically, although only probes to the mbr were used.

Molecular methods have proved to be useful in the identification of bcl-2 rearrangements when cytogenetic analysis reveals 14q+ or 18q- marker chromosomes only (Lee et al, 1987b; Lipford et al, 1987). This was not seen in this series, where rearrangements of bcl-2 were not detected molecularly in the 7 cases where abnormalities of chromosomes 14 and 18 other than t(14;18) were present.

There was also no clear demonstration of occult t(14;18) detected molecularly in the absence of cytogenetic evidence of the translocation. Lee et al (1987b) demonstrated submicroscopic bcl-2 rearrangements in 3/11 patients with
lymphoma who had cytogenetic abnormalities other than t(14;18). Two of the 3 had rearrangements detectable after digestion with more than one restriction enzyme (BamH1 and EcoRl) but none showed comigration with fragments detected by the JH probe.

In this series, one case with clonal karyotypic abnormalities other than t(14;18) showed a rearranged band after digestion with Bcl-1 restriction enzyme only and Southern blotting with the mbr probe. Failure to demonstrate rearrangements of bcl-2 by digestion with conventional enzymes such as BamH1 and EcoRl and the absence of comigration with fragments detected by the JH probe suggested that the presence of an occult but typical t(14;18) was unlikely. It is possible that the breakpoints within the immunoglobulin heavy chain locus lay elsewhere but further probing with probes to the constant, switch and variable regions was not performed.

The polymerase chain reaction proved the least reliable method for detecting t(14;18) in diagnostic specimens, giving an incidence of t(14;18) of only 48%. Those translocations not detectable by Southern blotting were also undetectable by polymerase chain amplification, again suggesting the breakpoints were outwith the even more restricted area covered by the PCR primers.
The unreliability of PCR in demonstrating bcl-2 rearrangements was mainly due to problems in amplifying breakpoints at the mcr. Almost all (90%) of the translocations involving the major breakpoint cluster region were detectable by PCR with only 2 having breakpoints outwith the amplified region. However, amplification by PCR of those translocations falling within the minor cluster region on Southern blotting detected only 2 of 9. Interestingly, in contrast to the mbr, only 4 had a band detectable by more than one restriction enzyme on Southern blotting and 6 failed to show comigrating bands on reprobing with the JH probe. Cleary et al (1986b) also described a difference in the pattern of Southern blotting using the same probe to the mcr and proposed that two breakpoint clusters may exist within the region of the mcr. Using the same mcr primers as used in this series, 2 of 10 translocations detectable at the mcr by Southern blotting were not amplifiable by PCR (Ngan et al,1989). Pezzella et al (1990a) also found that one of three minor breakpoints were not detected by PCR.

Reliable detection of mcr breakpoints may therefore require the use of more than one set of PCR primers. Failure to amplify the translocation by PCR can also be due to technical problems but all specimens were repeated on several occasions, with positive controls included on each run. It is conceivable that the DNA in some specimens was not suitable
for amplification but simultaneous amplification of a marker gene such as that for beta-globin was not carried out.

Few reports have been previously published on the distribution of breakpoints within bcl-2. The results of this series are very similar to those of Weiss et al (1987) who found that two thirds of breakpoints detectable by Southern blotting fell within the mbr and the remainder within the mcr. In contrast, Pezzella et al (1990a) reported a ratio of 6:1 in their series of 51 cases. However, almost 50% of these were studied by PCR alone, using mcr primers identical to those used in this series. The unreliability of amplification at the mcr demonstrated by the current results may account for the lower incidence of breakpoints detected by them in this region.

The molecular stability of t(14;18) was demonstrated by the identical banding pattern achieved on molecular analysis of material from two cases of lymphoma relapse. Lipford et al (1987) also showed that the breakpoints of t(14;18) were unaltered even when follicular lymphoma evolved into more aggressive diffuse disease.

4.6 Conclusions

The overall detection of t(14;18) in this series of 49 patients analysed by cytogenetics, Southern blotting and PCR was \( \frac{38}{49} \) (77%) (Table 4.2) which is greater than that
Table 4.2 Comparison of detection of t(14;18) in 49 patients with follicular lymphoma studied by cytogenetics, conventional electrophoresis (CE) and PCR. The number of positive cases for each technique is shown and also the site of the breakpoint as determined by molecular methods. mbr=major breakpoint region; mcr=minor cluster region.

<table>
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<th>mcr</th>
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<td>21/44(48%)</td>
<td>9/44(20%)</td>
<td>30/44(68%)</td>
</tr>
<tr>
<td>CE</td>
<td>22/49(44%)</td>
<td>2/49(4%)</td>
<td>24/49(48%)</td>
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<tr>
<td>Overall incidence</td>
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postulated for European lymphoma by Pezzella et al. (1990a) using molecular methods only. Of those bcl-2 rearrangements detectable by Southern blotting, 70% of breakpoints fell within the mbr, the remainder within the mcr. Although the most rapid technique, detection of t(14;18) by PCR amplification of diagnostic specimens from patients with follicular lymphoma proved the most inaccurate method. Almost 30% of translocations present cytogenetically (especially those at the mcr) were missed by this technique. The use of more than one set of primers at the mcr may be necessary to improve amplification of those translocations. The combined use of cytogenetics, conventional electrophoresis and PCR provided the most comprehensive means of detecting t(14;18) in this study but the limitations of all three techniques suggest that there possibly remains a small percentage of t(14;18) translocations which are undetectable by all three methods used. A recently published study supports this suggestion. Zelenetz et al. (1991) found that pulsed-field gel electrophoresis (PFGE) was superior to cytogenetics, CE and PCR in demonstrating t(14;18). PFGE utilises very large fragments of DNA (>50kb to 10,000kb) which minimises the risk that the restriction enzyme cleavage sites will fall outwith the areas covered by the molecular probes. Cytogenetics, CE and PCR detected t(14;18) in 24/29 cases of follicular lymphoma but PFGE detected a further case which was otherwise negative for t(14;18). PFGE detected t(14;18) in 8/12 cases which were
negative by Southern blotting and in 4/8 cases where the translocation was undetectable cytogenetically. These workers also confirmed the inferior detection rate of PCR which made it unsuitable for initial diagnostic screening. However, 10% of all cases remained negative for t(14;18) by all methods and this may represent the true t(14;18) negative population in follicular lymphoma.
CHAPTER 5

DETECTION OF MINIMAL DISEASE BY
PCR AMPLIFICATION OF t(14;18)
5.1 Introduction

Morphologic examination of the bone marrow will only detect the presence of lymphoma if malignant cells comprise 5% of the total, and sensitivity is undoubtedly less in follicular lymphoma where the malignant cells may appear histologically normal. Using newer techniques such as flow cytometry, immunophenotyping to detect clonal excess and Southern blotting to demonstrate gene rearrangements, sensitivity is increased to 1% (Berliner et al, 1986). This is often not adequate for the detection of minimal disease at diagnosis or residual disease after chemotherapy. However, the sensitivity of DNA amplification by the polymerase chain reaction allows detection of one malignant cell amongst 100,000 (Lee et al, 1987a).

In patients with follicular lymphoma, PCR amplification has detected t(14;18) bearing cells at diagnosis and after treatment when such cells were undetectable by conventional morphologic and immunologic methods (Lee et al, 1987a; Crescenzi et al, 1988; Stetler-Stevenson et al, 1988; Kneba et al, 1990). PCR detection of t(14;18) has also been used to monitor the effectiveness of ex-vivo purging of bone marrow prior to transplant (Negrin et al, 1991; Gribben et al, 1991a). The presence of minimal disease may have implications for
staging of lymphoma and therefore subsequent treatment and may also be important in monitoring response to therapy and predicting disease relapse.

In this study, the presence of minimal malignant disease was determined at diagnosis, relapse and after treatment in a series of patients with follicular lymphoma. DNA extracted from staging marrows, peripheral blood, post treatment marrows and peripheral blood stem cell harvests was amplified by PCR and the presence or absence of t(14;18) bearing cells determined. The significance of occult lymphoma is discussed.

5.2 Patient population and tumour characteristics

Eleven patients who had cytogenetic and molecular evidence of t(14;18) were identified retrospectively from the results described in Chapters 3 and 4. All had translocations which were amplifiable by PCR. Marrow or peripheral blood samples from each at time of diagnosis or relapse had been stored at -70°C for subsequent DNA analysis. The presence of lymphoma in marrow aspirates and biopsy specimens was determined by conventional morphological criteria.

Case 49 had two marrow samples stored, removed within one month of each other and both before any treatment was instituted. Two patients had peripheral blood stem cells harvested during chemotherapy (Craig et al, in press), and
one, Case 23, went on to have an autologous stem cell transplant. Samples of marrow, blood and stem cell harvests were stored from both cases during the course of their disease.

5.3 Method

Purified genomic DNA was extracted as described in Section 2.3. Samples were frequently small, consisting of 1-5x10^6 cells, and yielded insufficient DNA for conventional electrophoresis. DNA amplification was carried out as described in Section 2.10, using consensus JH primers and the primer appropriate to the breakpoint within bcl-2, as determined by Southern blotting of diagnostic lymph nodes. Where possible, several separate amplifications of each sample were performed. Both positive and negative controls as described in Section 4.3.2 were included in each amplification round and precautions taken to prevent contamination. Concomitant amplification of a marker gene such as beta globin to ensure DNA was suitable for PCR was not performed.

PCR products were visualised by ethidium bromide staining of 2% agarose gels. Positive bands were compared in size with those detected after amplification of DNA obtained from diagnostic lymph nodes.
5.4 Results

The results are summarised in Table 5.1, which compares histological findings with those of PCR. The results from those with more than one sample are arranged in chronological order. Negative control samples showed no evidence of specific bands after amplification. Circulating lymphoma cells were not detected in the three samples of peripheral blood examined.

Of the ten cases which had marrow available at diagnosis or relapse, seven had evidence of t(14;18) positive cells after PCR amplification. As expected, where histology suggested involvement of marrow by lymphoma, PCR confirmed the presence of disease. PCR also detected lymphomatous involvement in one marrow which was histologically negative (Case 52).

Interestingly, Case 49, which had two samples of marrow available, had t(14;18) detectable in one sample which was histologically positive. The second sample, taken one month later, but before treatment, was PCR negative. Histology was not available.

In case 23, in which samples are listed chronologically, marrow involvement by lymphoma was present at diagnosis but subsequent samples after treatment showed no evidence of t(14;18) positive cells after PCR amplification.
Table 5.1. Comparison of histological appearance and presence of occult lymphoma, as determined by PCR amplification of t(14;18). ABMT = autologous marrow transplant. SCH = peripheral blood stem-cell harvest. ND=not done; NK=not known.

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<td>+ve</td>
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<tr>
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Amplification of DNA extracted from peripheral blood stem cell harvests showed no evidence of t(14;18) containing cells. However, a marrow obtained after autologous stem cell transplant was positive at a time when the patient was in clinical remission.

Case 62 had histological evidence of marrow involvement by lymphoma at diagnosis and PCR amplification of a subsequent marrow at time of clinical remission detected t(14;18) carrying cells. A peripheral blood stem cell harvest was also positive. The patient subsequently relapsed with nodal and marrow disease.

5.5 Discussion

This small study confirms the usefulness of PCR in detecting minimal disease in those patients with t(14;18) and amplifiable breakpoints. Polymerase chain amplification of the hybrid DNA sequences of t(14;18) demonstrated the translocation in samples of marrow which were insufficient for conventional electrophoresis and which had not been submitted for cytogenetics. Not surprisingly, where marrow samples were histologically involved with lymphoma, PCR was positive also.

In a large series of 152 patients with NHL, Gribben et al (1991b) showed no advantage of PCR over histologic detection of low grade lymphoma at diagnosis, but all patients
were known to have advanced disease. Only 11% of patients had staging altered from III to IV on the basis of PCR detection of marrow involvement.

In this small series, the increased sensitivity of PCR detected malignant cells in three samples of marrow which were histologically normal. Case 52, analysed at relapse, became stage IV from III after PCR detected t(14;18). The presence of occult follicular lymphoma after treatment in patients otherwise thought to be in clinical remission is demonstrated by cases 23 and 62. This has been described sporadically by several groups (Stetler- Stevenson et al, 1988; Kneba et al, 1990) but was most convincingly described by Gribben et al (1991b) who found PCR evidence of t(14;18) in bone marrow samples after treatment in 100% of over 100 patients with low-grade lymphoma and an amplifiable translocation, although only 50% had morphologically evident disease.

However, case 49 demonstrates one of the problems of the limited marrow examination in follicular lymphoma provided by a single marrow aspirate. Two samples of marrow, taken one month apart, but before any treatment was instituted, gave conflicting results after PCR amplification. The first sample was histologically and PCR positive for lymphoma and the second was PCR negative, histology unknown. These samples were taken at diagnosis, when disease might be expected to be readily detected by PCR and the opposing
results probably reflect the patchy nature of lymphomatous infiltration of bone marrow. The situation is exacerbated after treatment where marked heterogeneity in PCR positivity from multiple bone marrow samples from individual patients has been observed (Gribben et al, 1991b). These authors recommend that at least two aspirates or biopsies are required to assess more accurately marrow involvement in follicular lymphoma.

Bearing in mind the limitations of a single sample in predicting marrow involvement, serial analysis of case 23 demonstrated marrow infiltration at diagnosis with subsequent samples becoming PCR negative for t(14;18) positive cells as treatment progressed. Peripheral blood stem cells obtained when the patient was receiving chemotherapy also appeared free of contaminating tumour cells and the patient underwent successful autologous transplantation using cryopreserved harvests. A marrow performed after transplant revealed the presence of occult lymphoma after PCR amplification although the patient remained in clinical remission.

The significance of such minimal disease in lymphoma has still to be established. In chronic myeloid leukaemia, after potentially curative allogeneic bone marrow transplantation, cytogenetic and molecular evidence of t(9;22)(q34;q11) may persist in marrow for many years before haematological or clinical relapse (Arthur et al, 1988; Arnold et al, 1989).
At the present moment, insufficient data exists in follicular lymphoma to assess the consequences of persistent or recurrent minimal marrow disease. The occurrence of t(14;18) at the early pre-B cell stage of development has led some workers to suggest that residual t(14;18) positive cells in bone marrow may represent a clonal population of cells which lack the subsequent genomic defects which give rise to follicular lymphoma (Crescenzi et al, 1988). Bertoli et al (1988) demonstrated that several transformational events were required before nodal lymphoma evolved from an oligoclonal pool of preneoplastic cells in the bone marrow.

However, patients with histologically evident marrow involvement at the time of autologous bone marrow transplant for follicular lymphoma had a significantly shorter disease-free survival than those in whom marrow was morphologically free of tumour (Freedman et al, 1991), suggesting that residual disease may play a role in relapse. Similarly, the detection by PCR of t(14;18) positive cells in marrow after immunologic purging prior to autologous transplantation was the most important prognostic indicator in predicting relapse (Gribben et al, 1991a). As expected therefore, case 23 remains in haematologic remission despite the presence of occult lymphoma. In contrast, case 62, who had histological evidence of marrow involvement at diagnosis, had persistently detectable t(14;18) positive cells in marrow and stem cell
harvests and eventually succumbed to overt nodal and marrow relapse.

5.6 Conclusions

These results confirm the increased sensitivity of PCR amplification in the detection of t(14;18) bearing cells both in diagnostic marrows and after treatment. Where the marrow is morphologically involved with lymphoma at diagnosis, PCR amplification of t(14;18) provides no additional information. PCR is, however, extremely useful in the detection of minimal disease after treatment. Because of its extreme sensitivity and requirement for micrograms or less of DNA the technique has considerable advantages over conventional electrophoresis or immunophenotyping. The patchy nature of lymphomatous infiltration of marrow after treatment may produce false negative results and it is recommended that more than one aspirate or biopsy is examined.

PCR was able to detect residual malignant cells in peripheral blood stem cell harvests, where minimal residual disease would be undetected by standard cytogenetic and immunologic techniques. As autologous transplantation becomes more prevalent in the treatment of follicular lymphoma, PCR will become crucial in the detection of residual malignant cells bearing t(14;18). Early results have already suggested that the inability to purge residual lymphoma cells
from marrow prior to reinfusion may be an important prognostic indicator of subsequent relapse (Gribben et al, 1991a). It is not yet clear whether these residual cells contribute to relapse or are simply evidence of more aggressive, refractory disease.
CHAPTER 6

CONCLUSIONS
This study of 72 patients with follicular lymphoma is one of the largest studies from a single centre in which the incidence of t(14;18) was determined by cytogenetic analysis, conventional electrophoresis and DNA amplification by the polymerase chain reaction. The translocation was detected in 55/72 (76%) of cases which exceeds the incidence proposed by Pezzella et al (1990a) for European lymphoma and approaches that of large American series which used molecular methods only (Weiss et al, 1987). Cytogenetic analysis of diagnostic specimens proved the most reliable technique for detecting the translocation and also provided additional karyotypic information of possible diagnostic and prognostic significance.

Southern blotting was almost as successful as cytogenetics but failed to detect cytogenetically visible translocations in three patients. Disappointingly, the rapid and highly sensitive PCR technique was the least successful, with over 30% of cytogenetically evident translocations being missed. The majority of failures were associated with breakpoints at the minor cluster region and the use of other primers from that region may improve detection.

Molecular methods were able to detect t(14;18) in diagnostic specimens where cytogenetics had failed but did
not reveal sub-microscopic bcl-2 rearrangements as reported by others (Lee et al, 1987b). That is perhaps not surprising as such rearrangements are rare and only a small number of cases lacking t(14;18) cytogenetically were studied in this series.

It is therefore recommended that all three techniques are required to most accurately determine the incidence of t(14;18) in follicular lymphoma. Similar conclusions were recently published (Zelenetz et al, 1991) but these authors also found that pulsed-field gel electrophoresis was the most comprehensive method of detecting t(14;18) and that some translocations are only detectable by this technique.

Both t(14;18) and bcl-2 rearrangements detected molecularly provided stable clonal markers of disease in the small number of patients analysed more than once in this study. Caution is advised in the interpretation of negative results after PCR amplification when disease is minimal as residual disease is often patchy and more than one sample is required.

The numerous other karyotypic abnormalities found in follicular lymphoma have also been discussed in this thesis. At present, in Edinburgh, these cytogenetic results are of research interest only and play no part in the management of patients with follicular lymphoma. Other factors such as age, stage, histologic subtype and the presence of bulk
disease are much more important factors in planning treatment and determining survival. Many of the published results on prognostic karyotypic abnormalities in follicular lymphoma are conflicting: the finding of extremely complex genetic lesions at diagnosis in the diverse group of patients with follicular lymphoma has made it almost impossible to assign accurately prognostic significance to a particular cytogenetic abnormality.

Perhaps the most useful role of t(14;18) in follicular lymphoma is as a stable molecular disease marker, detected initially by PCR and used in further staging, in the monitoring of response to treatment and in the detection of relapsed or residual disease. It is likely that those who would benefit most from this approach are the young, with aggressive refractory lymphoma. In such patients, PCR detection of residual malignant cells bearing t(14;18) after treatment, in autologous marrow after purging or peripheral blood stem cell harvests may be crucial in determining further treatment and prognosis.
CHAPTER 7

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