MOLECULAR CYTOGENETIC STUDIES IN THE HODGKIN AND REED-STERNBERG CELLS OF CLASSICAL HODGKIN LYMPHOMA

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I declare that this is entirely my own composition and that the studies described herein are the results of my own work, except where otherwise acknowledged.

ABBREVIATIONS

ALCL	Anaplastic large cell lymphoma
ALK	Anaplastic lymphoma kinase
ALL	Acute lymphoblastic leukemia
BAC	Bacterial artificial chromosome
BCL	B-cell lymphoma/leukemia
BL	Burkitt´s lymphoma
BLAST	Basic Local Alignment Search Tool
BM	Bone marrow
bp	Base pairs
CCD	Charge coupled device
CD	Cluster of differentiation
CEP	Centromeric probe
CGH	Comparative genomic hybridisation
cHL	Classical Hodgkin lymphoma
CLL	Chronic lymphocytic leukaemia
CoT-1	Concentration over time 1
DAPI	Diamidino phenylindole
dATP	2´- Deoxyadenosine 5´-triphosphate
dCTP	2´- Deoxycytidine 5´-triphosphate
dGTP	2´- Deoxyguanosine 5´-triphosphate
DLBCL	Diffuse large B-cell lymphoma
DNA	Deoxyribonucleic acid
dNTP	2´-Deoxynucleoside 5´-triphosphate
DOP	Degenerate oligonucleotide primed
dTTP	2´- Deoxythymidine 5´-triphosphate
dUTP	2´- Deoxyuridine 5´-triphosphate
EBER	Epstein-Barr virus small polymerase III transcript
EBV	Epstein-Barr virus
EBNA	Epstein-Barr virus nuclear antigen

ESR	Erythrocyte sedimentary rate
FACS	Fluorescence activated cell sorting
FCL	Follicle centre cell lymphoma
FICTION	Fluorescence immunophenotyping and interphase cytogenetics as a tool for the investigation of neoplasms
FISH	Fluorescence *in situ* hybridisation
FRET	Fluorescence resonance energy transfer
HD	Hodgkin´s disease
HGP	Human genome project
HHV	Human herpes virus
HL	Hodgkin lymphoma
HRS	Hodgkin and Reed-Sternberg
ICC	Immunocytochemistry
Ig	Immunoglobulin
IgH	Immunoglobulin heavy chain
IgL	Immunoglobulin light chain
IkB	Inhibitor of nuclear factor kappa B
IKK	IκB kinases
IL	Interleukin
IL-2	Interleukin-2
IM	Infectious mononucleosis
JNK	c-jun N-terminal kinase
kb	Kilobase
L&H	Lymphocytic and histiocytic
LDHL	Lymphocyte depleted HL
LCL	Lymphoblastic cell line
LDH	Lactate dehydrogenase
LMD	Laser microdissection
LMP1	Latent membrane protein 1
LOH	Loss of heterozygosity
LRHL	Lymphocyte rich HL

MAP	Mitogen-activated protein
MCHL	Mixed cellularity HL
MCL	Mantle cell lymphoma
MDA	Multiple displacement amplification
MEKK	Mitogen-activated protein kinase/ERK kinase kinase
MYC	v-myc avian myelocytomatosis viral oncogene homologue
NAP	Sodium purification
NCBI	National Center for Biotechnology Information
NF-kB	Nuclear factor kappa B
NHL	Non-Hodgkin lymphoma
NIK	NF-κB-inducing kinase
NLPHL	Nodular lymphocyte predominant HL
NPM	Nucleophosmin
NSHL	Nodular sclerosis HL
OD	Optical density
PAC	P1-derived artificial chromosome
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
PEN	Polyethylene naphthalate
PEP	primer extension pre-amplification
PIN	Prostatic intraepithelial neoplasia
PMBCL	Primary mediastinal B cell lymphoma
REAL	Revised European-American classification of lymphoid neoplasms
REL	v-rel avian reticuloendotheliosis viral oncogene homologue
RHR	Rel homology region
RPCI	Roswell Park Cancer Institute
RS	Reed-Sternberg
RT	Room temperature
RT-PCR	Reverse transcriptase polymerase chain reaction
SCID	Severe combined immunodeficient mice
SDS	Sodium dodecyl sulphate

SG	Spectrum green
SNP	Single nucleotide pleomorphism
SO	Spectrum orange
SRD	Signal response domain
TARC	Thymus and activation regulated chemokine
TCR	T-cell receptor
TGF	Transforming growth factor
TNFR	Tumour necrosis factor receptor
TNF	Tumour necrosis factor
TRAF	TNFR-associated factor
UV	Ultra-violet
WGA	Whole genome amplification
WHO	World Health Organization

ABSTRACT

Hodgkin lymphoma (HL) is a malignant lymphoma that largely affects young adults and is the second most common malignancy in this age group. Overall, around one third of cases are associated with the Epstein-Barr virus (EBV) and this association is believed to be causal. In young adults less than 20% of cases are EBV-associated and there are few clues to the aetiology of the EBV-negative form of the disease. Studies on the molecular genetics of HL have been hindered by the scarcity of the tumour cells, the Hodgkin and Reed-Sternberg (HRS) cells, within tumours and the lack of an animal model. Recently developed techniques for the enrichment and micromanipulation of HRS cells from cell suspensions and frozen sections have, however, opened up new avenues for the investigation of this enigmatic disease.

Diagnosis of HL is often straightforward but in some cases differentiation from non-Hodgkin lymphoma (NHL) can be problematic. In a recent epidemiological study of 575 consecutive cases of HL in Scotland and the Northern Region of England, expert histopathological review suggested that ~5% of cases had been wrongly classified as HL (Jarrett et al. 2003). Although there has been enormous progress in the treatment of HL around 25% of cases do not respond to conventional treatment, and there is a need for better prognostic markers to identify such patients at the time of diagnosis in order to institute more aggressive therapy.

Cytogenetic analysis has been extremely productive in the investigation of NHLs where the identification of non-random chromosomal abnormalities has led to the discovery of numerous oncogenes. In follicle centre cell lymphoma (FCL), tumour cells carry the t(14:18) translocation which juxtaposes the immunoglobulin (Ig) heavy chain gene on chromosome 14 with the bcl-2 gene on chromosome 18, resulting in deregulation of BCL-2. Investigation of this translocation has led to an improved understanding of the biology of both FCL and apoptosis, and the demonstration of t(14;18) has proven useful both diagnostically and in clinical follow-up. Similarly, the identification of the t(2:5) translocation in anaplastic large cell lymphoma (ALCL) allowed production of antibodies to the fusion protein NPM-ALK and the recognition of a discrete subgroup of patients with ALK-positive lymphoma or 'ALKoma'.

In contrast to the situation with NHL, conventional cytogenetic studies in HL have been less numerous and have been hindered by the scarcity and low mitotic rate of HRS cells. Normal karyotypes have been found in almost half the cases studied but it is questionable whether such karyotypes actually correspond to the HRS cells. In addition, chromosomal abnormalities have been detected in the reactive component of lesions further complicating interpretation.

An alternative approach to studying genetic abnormalities in HL is to use comparative genomic hybridisation (CGH). This technique provides a global approach for screening clinical specimens for genetic imbalances and can be performed when only very small amounts of tumour deoxyribonucleic acid (DNA)

are available. CGH involves labelling tumour DNA and DNA from a normal source with two different fluorescent dyes. The labelled DNA samples are mixed at an equal ratio and used as a probe for *in situ* hybridisation on normal metaphase spreads. The hybridisation profile of test and control DNA along the length of each chromosome is then analysed, the fluorescent ratio of the two fluorochromes is calculated and regions of chromosomal loss or gain determined. Two groups have recently applied CGH to the study of HL. Using slightly different methodologies, both found consistent chromosomal losses and gains, however, the results from the two studies differed, emphasising the need for additional studies.

To determine whether recurrent genomic imbalances are a feature of HL, CD30-positive HRS cells were laser microdissected from 20 cHL cases and 4 HL-derived cells lines and subjected to analyses by CGH. In primary tumours, the most frequently involved chromosomal gains were 17q (70%), 2p (40%), 12q (40%), 17p (40%), 22q (35%), 9p (30%), 14q (30%), 16p (30%), with minimal overlapping regions at 17q21, 2p23-13, 12q24, 17p13, 22q13, 9p24-23, 14q32, 16p13.3 and 16p11.2. The most frequent losses involved 13q (35%), 6q (30%), 11q (25%) and 4q (25%), with corresponding minimal overlapping regions at 13q21, 6q22, 11q22 and 4q32. Statistical analysis revealed significantly more gains of 2p and 14q in the older adult cases; loss of 13q was associated with a poor outcome. The results suggest that there is a set of recurrent chromosomal abnormalities associated with cHL and provide further evidence that cHL is genetically distinct from nodular lymphocyte predominance Hodgkin lymphoma (NLPHL). Abnormalities of 17q are infrequent in other lymphoma or NLPHL; this finding,

coupled with current knowledge of gene expression in cHL, suggests that genes present on 17q may play an important role in the pathogenesis of cHL. Combined immunophenotype and interphase cytogenetic (FICTION) studies were used as techniques for follow-up studies.

High expression of both STAT3 and STAT5a has been described in cHL (Chen et al. 2001; Hinz et al. 2002) and their genes are located in 17q21.2. Constitutive activation of NF-κB has been found to be a feature of the HRS cells in cHL and has been shown to facilitate escape from apoptosis (Bargou et al. 1996). The c-rel gene encodes for a subunit of NF-κB and is located on chromosome 2p16. REL amplification has been shown in some cHL cases (Joos et al. 2002) and therefore REL was selected as another candidate gene for further study. Non-functional inhibitor proteins of NF-κB, such as IκBα, has been described as an alternative mechanism for the aberrant activation of NF-κB (Wu et al. 1996). As part of the follow-up studies, *IkBa* gene mutation status and loss of heterozygosity (LOH) in the HRS cells were determined by sequence analysis and SNP assays.

FICTION confirmed that gains of 2p involved the REL gene but gains on 17q were not due to amplification of STAT3/5a. Other candidate genes present on 17q could be important in the pathogenesis of cHL. Frequent *IkB*a mutations were detected but many are not considered to be of functional importance. REL gain, EBV status, *IkBa* mutation or LOH were not mutually exclusive mechanisms in the pathogenesis of cHL.

Chapter 1

GENERAL INTRODUCTION

1.1 Introduction

Hodgkin lymphoma (HL) is the commonest form of malignant lymphoma in young adults (Cartwright et al. 1997) and accounts for approximately 30% of all lymphoma. Although in recent years, cumulative evidence has pointed towards a causal relationship between the Epstein-Barr virus (EBV) and HL, this only applies to a proportion of cases (Gledhill et al. 1991; Jarrett et al. 2003). In young adults where the majority of cases are negative for EBV, the aetiology remains unknown. It is suspected that other infectious agents may be involved but so far they remain elusive (Jarrett and MacKenzie 1999).

In contrast to non-Hodgkin lymphoma (NHLs), which frequently arise at extranodal sites and spread in an unpredictable fashion, HL typically arises from cervical lymph nodes and spreads to anatomically contiguous nodes. HL is characterised morphologically by the presence of the distinctive neoplastic giant cells, Hodgkin and Reed-Sternberg (HRS) cells, which are always surrounded by a mixture of non-neoplastic inflammatory and accessory cells.

Studies on the molecular genetics and cytogenetics of HL have been hampered by the scarcity of HRS cells within lesions, which often make up only 0.1-1% of the cells within affected lymph nodes (Kapp et al. 1995), and the lack of model systems. There are currently 15 HL-derived cell lines but only one of these (L1236) is proven to be derived from the original tumour cells. Attempts at producing useful animal models have also been unsuccessful (Krajewski et al.

1995). Severe combined immunodeficient (SCID) mice injected with cell suspensions from HL nodes develop tumours that have a similar phenotype but not genotype to the original HRS cells, indicating that they are not derived from the tumour cells.

1.2 Clinical features of Hodgkin lymphoma

HL commonly presents with enlargement of the cervical lymph nodes, but nodes elsewhere may also be the primary site. Enlarged nodes are classically painless and 'rubbery'. Other clinical features of HL are fever, drenching night sweats, loss of weight, pruritus and alcohol-induced pain at the site of the enlarged node. Fever, night sweats and weight loss constitute the B symptoms, and are usually seen in more advanced stages of the disease. The classical Pel-Ebstein fever, which consists of a few days of high pyrexia followed by apyrexia for a few days, is uncommon. Symptoms due to involvement of other organs, for example, bone, lung and skin, may rarely be seen. Examination reveals lymphadenopathy, sometimes with hepatomegaly and splenomegaly depending on the stage of disease. A chest X-ray and/or chest CT may show mediastinal lymphadenopathy or pulmonary infiltration. The diagnosis of HL rests on the biopsy of a suitable node (Malpas 1990).

Following the diagnosis of HL, staging is carried out to determine the extent of the disease. The Ann Arbor staging classification for HL was formulated in 1971 to provide a rational basis upon which treatment decisions could be made for patients at initial presentation (Rosenberg et al. 1971). With the advent of better diagnostic

imaging and the recognition of important prognostic criteria, the classification was revised at a meeting in the Cotswolds, UK in 1988 (Lister and Crowther 1990). Abdominal CT scanning has replaced the use of staging laparotomy and is used to detect infiltration of iliac, para-aortic and coeliac nodes. PET scanning has recently been introduced and it has been used in clinical trial situations (Naumann et al. 2004).

In the early stages the haematological findings are usually normal. Later, a normochromic, normocytic anaemia with a raised erythrocyte sedimentary rate (ESR) is commonly seen. Marrow involvement is uncommon but may be associated with leucoerythroblastic anaemia. Biochemical findings include hyperuricaemia and abnormal liver biochemistry due to liver involvement. Raised lactate dehydrogenase (LDH) has been found by some groups to be associated with a poor prognosis but this association is not apparent in the large international datasets (Straus et al. 1990; Ferme et al. 1997).

Over the past 50 years, the outcome for patients with HL has changed from being almost invariably fatal to being curable in the majority of cases. The two effective treatments for HL are radiotherapy and combination chemotherapy. Despite the success of such therapies, the morbidity associated with the effects of treatment including cardiopulmonary fibrosis, infertility and secondary malignancy cannot be underestimated. Failure to achieve a complete response and early relapse are often associated with a bad prognosis, and there are few long-term survivors in this group. At present, there are no specific markers that can distinguish this

subgroup but prognostic indexes are sometimes useful (Proctor et al. 1991; Hasenclever 2002).

1.3 Histopathology of Hodgkin lymphoma

The diagnosis of cHL is established with the identification of Reed-Sternberg (RS) cells in the appropriate cellular milieu. RS cells are large cells that either have a large bi-lobed or multi-lobated nucleus (Figure 1.1). Each lobe or nucleus contains one large inclusion-like eosinophilic nucleolus, up to about 10 μm in size, the diameter of a small lymphocyte nucleus. The cytoplasm is usually relatively abundant, so that an overall diameter of 20 to 50 μm can be reached. Mononuclear Hodgkin cell variants are also present and with RS cells are collectively called HRS cells. Lacunar cells are mononuclear HRS cells with abundant amphophilic cytoplasm and retracted nuclei seen in formalin-fixed paraffin-embedded sections. Occasionally, apoptotic Hodgkin cells, sometimes termed mummified or zombie cells, are present (Chan 2000). Cells closely resembling HRS cells can be found in a wide variety of reactive and neoplastic diseases and therefore these cells must be present in the appropriate background before a diagnosis can be reached. The reactive infiltrate in the background surrounding the HRS cells is composed of a variable mixture of lymphocytes, eosinophils, neutrophils, plasma cells and histiocytes. The definitive diagnosis of HL can be made by morphological assessment alone although immunocytochemistry (ICC) is usually used to assist diagnosis and differentiate subtypes (Weiss et al. 1999; Stein et al. 2001; Zochowski et al. 2001). The HRS

cells typically express cluster of differentiation (CD) 15 and CD30 but lack B or T cell lineage markers (see section 1.5).

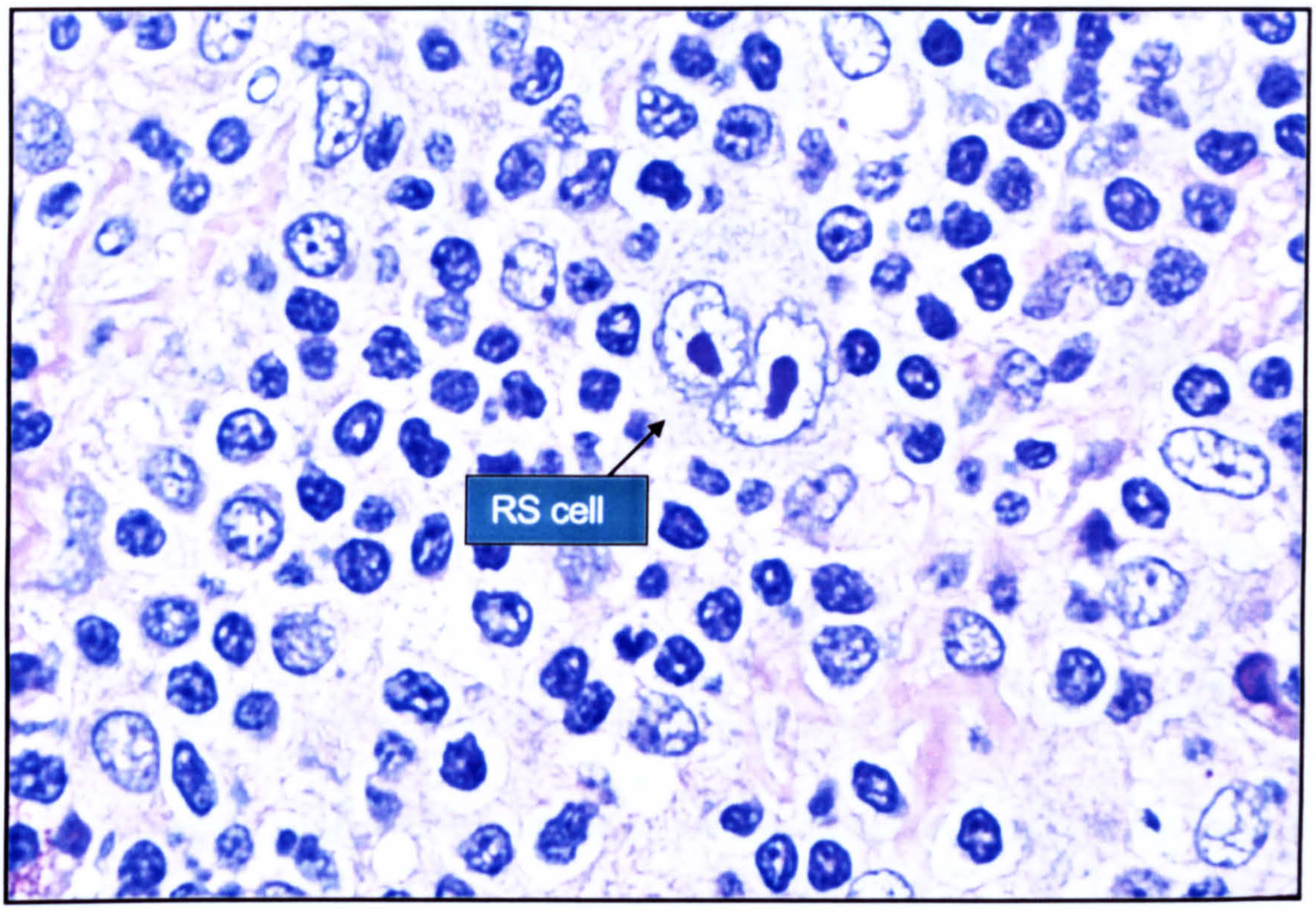

Figure 1.1 Image of Hodgkin and Reed-Sternberg cells from section of paraffin embedded material (x400, H&E staining, courtesy of Dr. J.K.C. Chan).

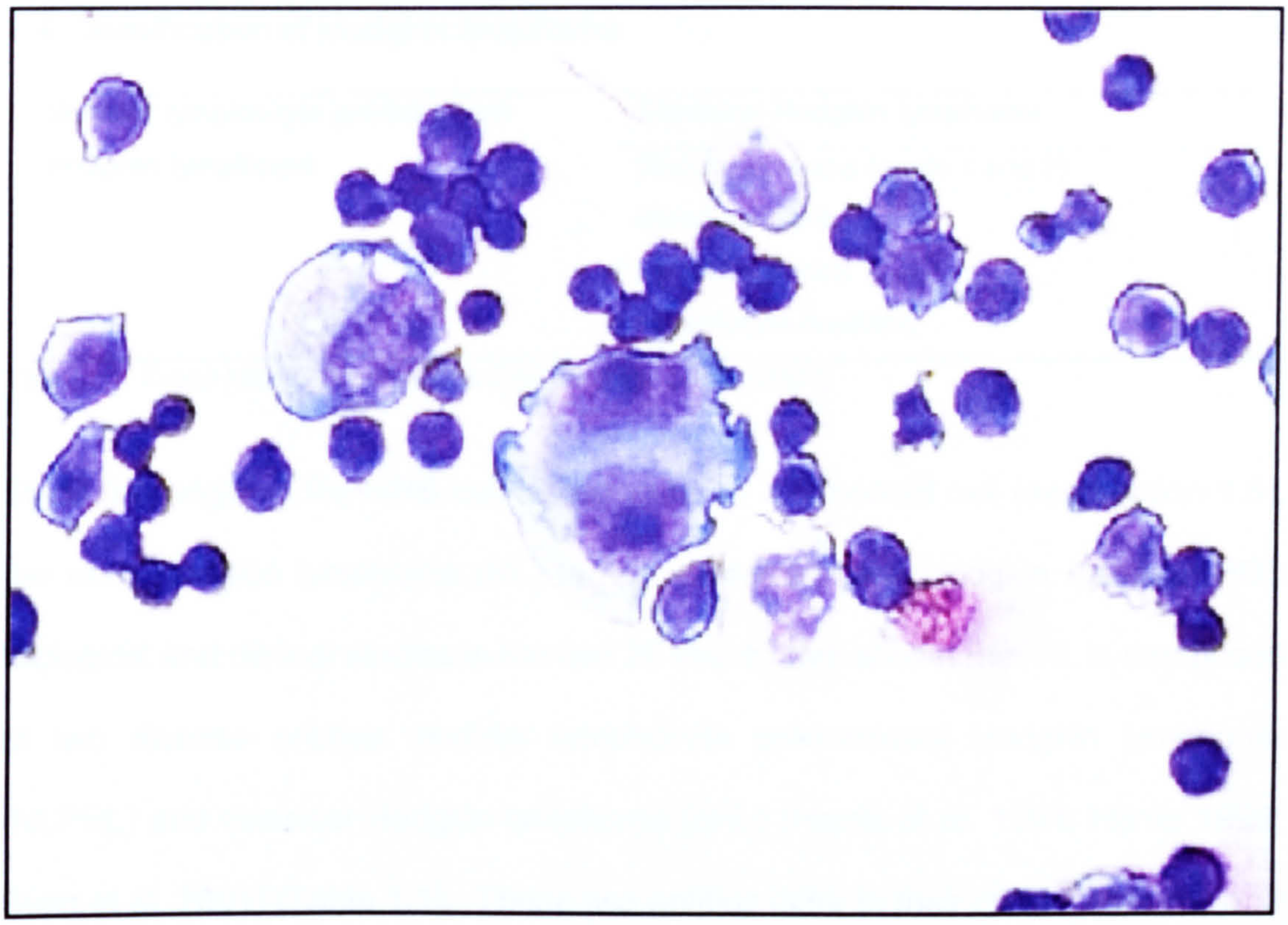

Figure 1.2 Image of Hodgkin and Reed-Sternberg cell from cytological preparation (x400, DiffQuik staining).

Cytology of HL (Figure 1.2) shows a dispersed population of lymphoid and other cells in which scattered large cells are evident (Das and Gupta 1990; Olson et al. 2000). As expected, the lymphocytes are small with a mature chromatin pattern. The HRS cells are recognisable as large cells with bilobed or multilobated nuclei and prominent nucleoli (Weiss et al. 1999). Such features were helpful for recognition of HRS cells in cytospin preparations used in this research project.

1.4 Classification of Hodgkin lymphoma

Nodular lymphocyte predominant Hodgkin lymphoma	Classical Hodgkin lymphoma
	Nodular sclerosis (grade 1 and 2)
	Mixed cellularity
	Lymphocyte-rich
	Lymphocyte-depleted

Table 1.1. World Health Organisation Classification of HL (2001)

Since the origin of the HRS cell is known to be a lymphoid cell (see section 1.5), the term Hodgkin lymphoma (HL) is now preferred over Hodgkin disease (HD). Biological and clinical studies in the last 20 years have shown that HL is comprised of two disease entities: nodular lymphocyte predominant Hodgkin lymphoma (NLPHL) and classical Hodgkin lymphoma (cHL) (Harris et al. 1994; Harris 1999; Stein et al. 2001) (Table 1.1). These two entities differ in their clinical features and behaviour, their histopathology and the origin of the tumour cells (see section 1.5). Four subtypes are described within classical Hodgkin lymphoma (cHL): nodular sclerosis (NS), mixed cellularity (MC), lymphocyte rich classical (LRC) and lymphocyte-depleted (LD). These subtypes differ somewhat in their clinical features, the composition of the cellular background, histopathological appearance and the frequency of EBV involvement, but not in the immunophenotype of the HRS cells, which is the same in all four variants.

1.4.1 Nodular lymphocyte predominant Hodgkin lymphoma

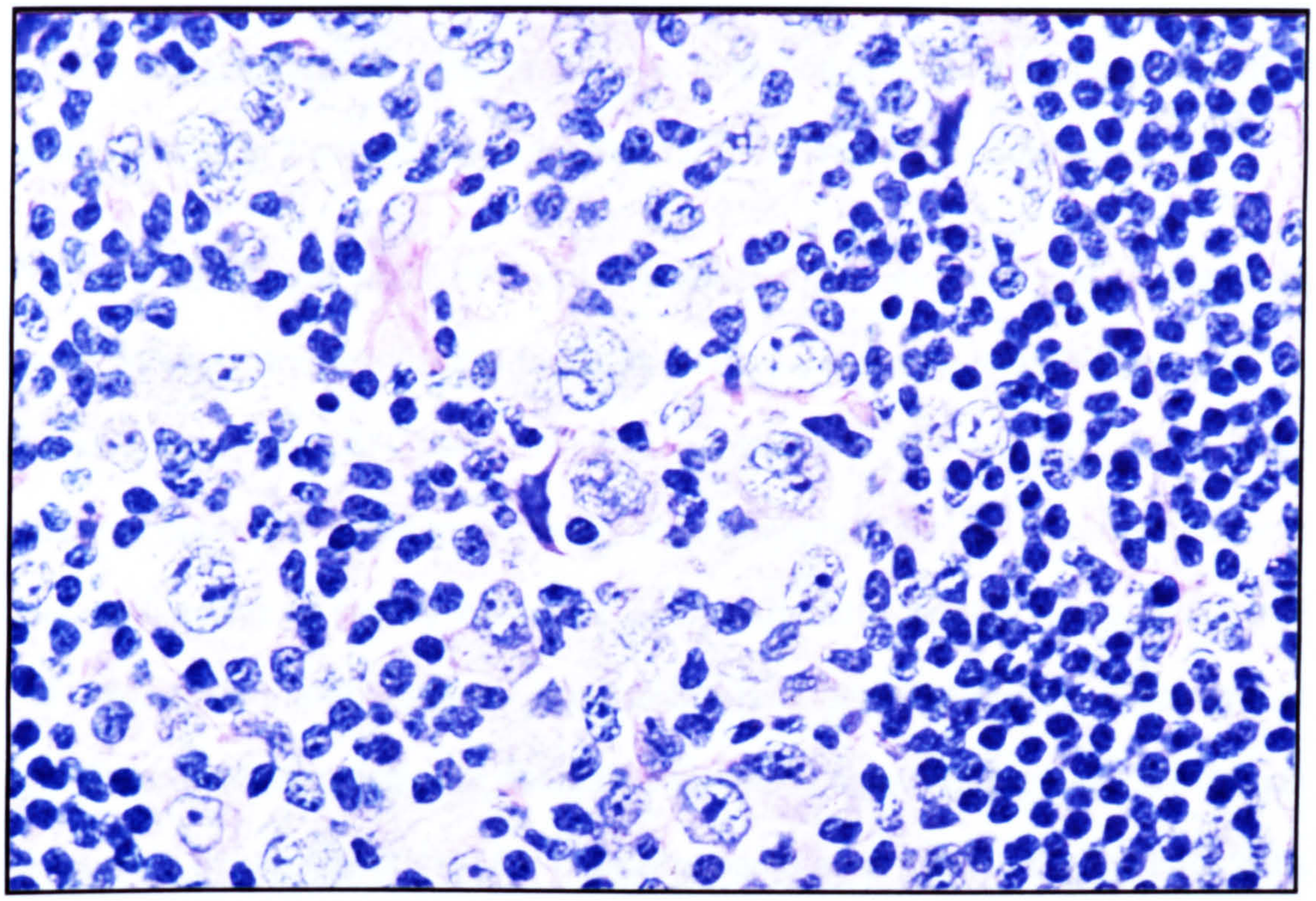

Figure 1.3 Image of L&H cells from section of paraffin embedded material (x400, H&E staining, courtesy of Dr. J.K.C. Chan).

At low magnification, the lymph node shows complete or subtotal effacement of the architecture. The capsule is usually intact without pericapsular infiltration and there may be a rim of uneffaced lymphoid tissue, which may be normal, hyperplastic or show progressive transformation of germinal centres. Fibrosis is uncommon but may be present and may be band-like, mimicking NSHL. A nodular or a nodular and partly diffuse infiltration is found in the effaced areas. The presence of a nodular component is mandatory for the diagnosis of NLPHL. Nodules are large and relatively numerous, sometimes resembling progressively

transformed germinal centres. In some cases the nodules are poorly demarcated and difficult to discern, and a diffuse architecture may be focally present. If a large number of well-prepared sections show no evidence of nodularity, the possibility of either LRCHL or T-cell-rich large B cell lymphoma should be considered.

The neoplastic cells of NLPHL are the lymphocytic and histiocytic (L&H) cells (popcorn cells) and are usually found in and around the nodules (Figure 1.3). In diffuse areas, the L&H cells resemble centroblasts but are larger and have lobulated nuclei and small to moderate-sized basophilic nucleoli. The cytoplasm is abundant and only slightly basophilic. Ultrastructural studies demonstrate that L&H cells have the appearance of centroblasts of germinal centres (Poppema et al. 1979). In addition, follicular dendritic cells characteristic of the B-cell follicle can be found in the vicinity of the L&H cells (Poppema et al. 1979). Typical HRS cells are few in number or completely lacking. If typical HRS cells are easily detected, a diagnosis of LRCHL should be suspected. In some cases, L&H cells might resemble lacunar cells because both cell types show irregularly shaped or lobulated nuclei, small nucleoli, and broad pale to slightly basophilic cytoplasm.

1.4.2 Nodular sclerosis Hodgkin lymphoma

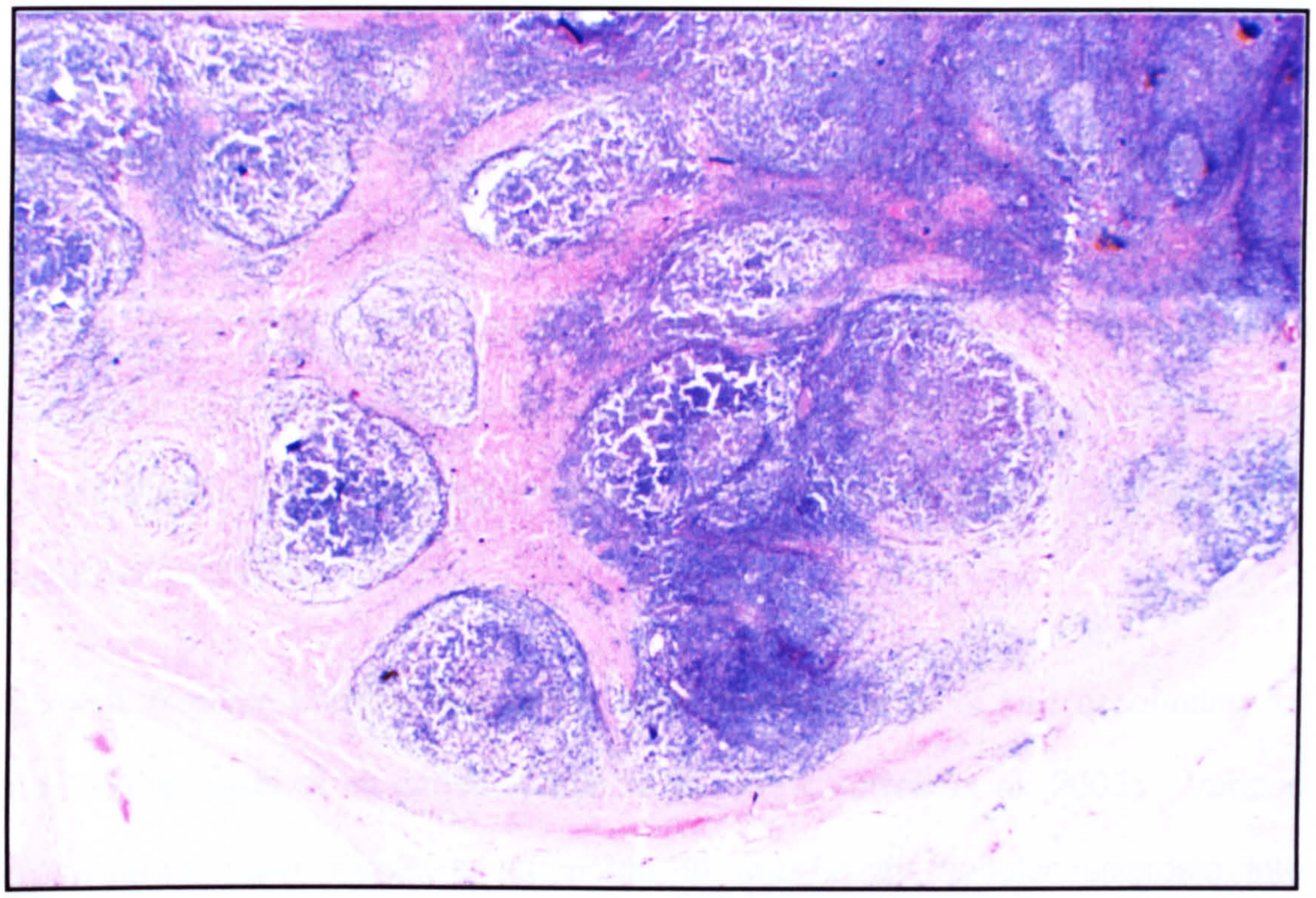

Figure 1.4 Image of NSHL from section of paraffin embedded material (x10, H&E staining, courtesy of Dr. J.K.C. Chan).

The NS subtype of cHL is characterized by collagenous bands and lacunar cells (Figure 1.4). The presence of one or more sclerotic bands is the defining feature. These bands usually radiate from a thickened lymph node capsule, often following the course of a penetrating artery. The bands are composed of mature, laminated, relatively acellular collagen and are birefringent in polarized light. In most cases, several broad collagenous bands can be identified, but a single band may be present, or fibrosis may be so extensive that only isolated nodules of lymphoid tissue remain.

The collagenous bands of NSHL enclose nodules of lymphoid tissue containing variable numbers of HRS cells. Lacunar cells are the most common type of HRS cell present and may be found in large numbers or in sheets. They tend to aggregate at the centre of nodules, sometimes forming a rim around central areas of necrosis. Classical RS cells are difficult to find and may not be found in small biopsy specimens. Eosinophils and neutrophils are often numerous, but histiocytes and plasma cells are usually less conspicuous in the NS subtype. Fibrohistiocytic foci are sometimes found in the centres of nodules or extensively replacing the tissue.

In most Western centres, NS is the most common type of HL, accounting for around two-thirds of all cases (Colby et al. 1981; Jarrett et al. 2003). Various investigators have therefore attempted to subclassify nodular sclerosis into prognostic groups. The British National Lymphoma Investigation (BNLI) has established a grading system for NSHL (MacLennan et al. 1992). In Grade 1, 75% or more of the nodules contain scattered HRS cells in a lymphocyte rich, mixed cellular, or fibrohistiocytic background. In Grade 2, at least 25% of the nodules contain increased numbers of HRS cells (defined as a sheet of cells filling a x400 high power field). Although this system has been criticised on the basis of reproducibility, (Ferry et al. 1993) have demonstrated that cases classified as Grade 2 have a significantly worse prognosis than those classified as Grade 1; (Hess et al. 1994) have failed to demonstrate a difference between these grades of NSHL. A more recent study has suggested a simpler grading system based on

number of eosinophils, lymphocyte depletion and cellular atypia (von Wasielewski et al. 2003).

1.4.3 Mixed cellularity Hodgkin lymphoma

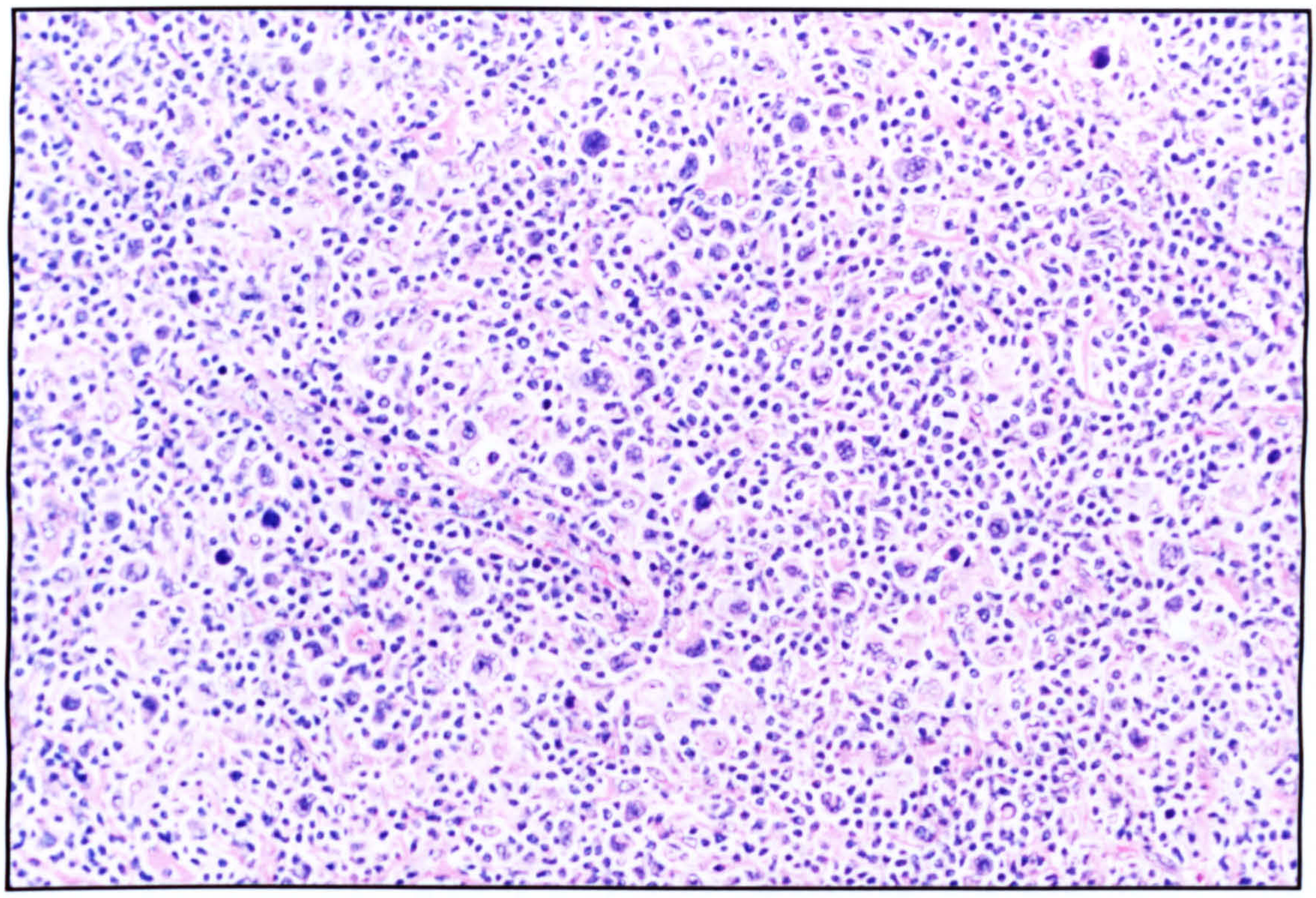

Figure 1.5 Image of MCHL from section of paraffin embedded material (x200, H&E staining, courtesy of Dr. J.K.C. Chan).

The MC subtype makes up approximately 21% of cases of HL in Western populations, but it may constitute 50% or more of the cases in developing countries (Correa and O'Conor 1971; Jarrett et al. 2003). The capsule is usually intact and of normal thickness. A vague nodularity may be present at low magnification, but the presence of any definite fibrous bands would warrant

classification as NS rather than MC. At high magnification, a heterogeneous mixture of HRS cells, small lymphocytes, eosinophils, neutrophils, epithelioid and non-epithelioid histiocytes, plasma cells, and fibroblasts is present (Figure 1.5). Classical RS cells are usually easy to find.

1.4.4 Lymphocyte-depleted Hodgkin lymphoma

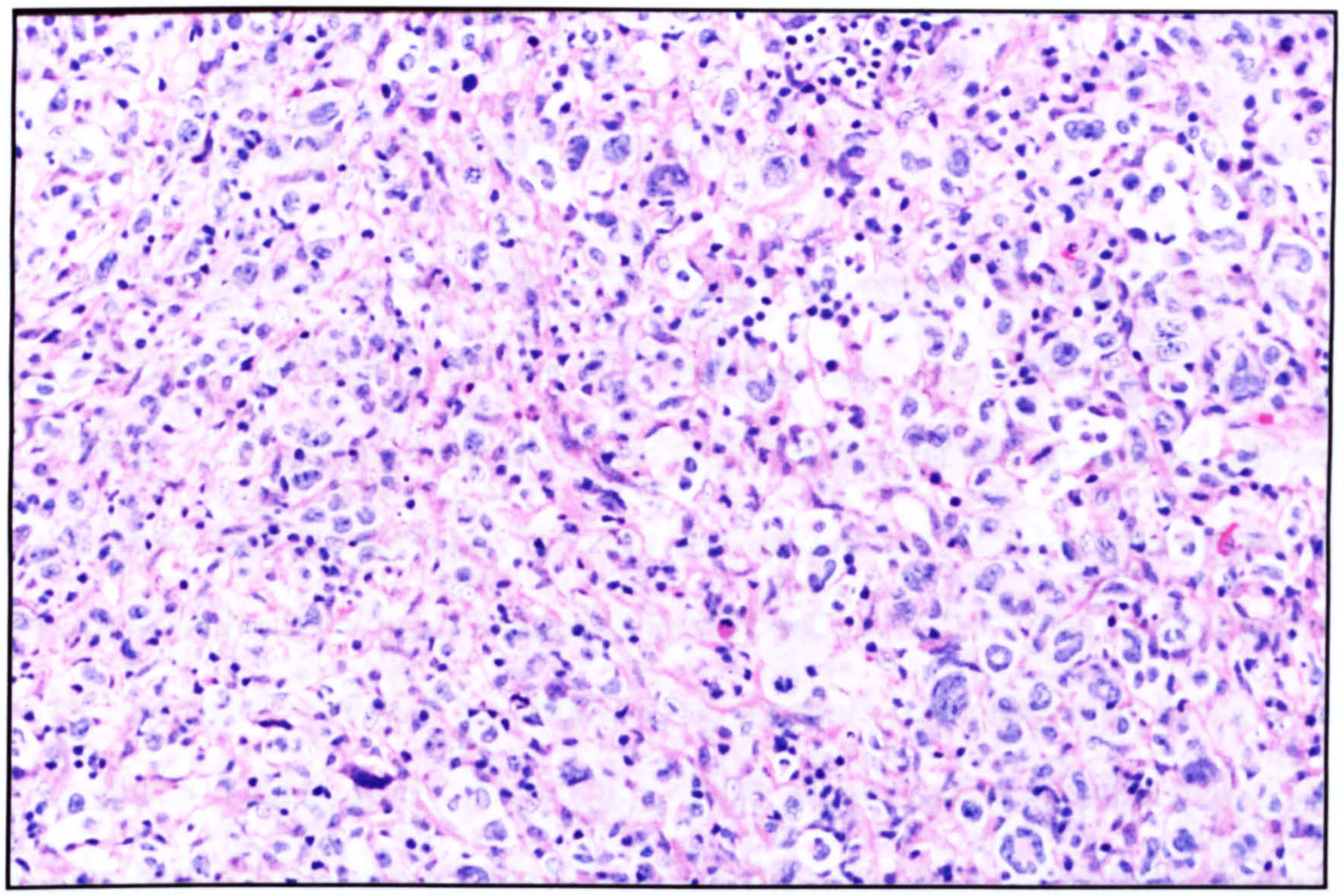

Figure 1.6 Image of LDHL from section of paraffin embedded material (x200, H&E staining, courtesy of Dr. J.K.C. Chan).

The LD subtype has almost disappeared with the use of modern classification systems. The most characteristic features are a marked degree of reticulin fibrosis surrounding single cells and lymphocyte depletion (Figure 1.6). In the reticular

variant, sheets of HRS cells, often showing pleomorphic features, are found. In contrast to NS, this subtype is not characterized by the presence of thick fibrous bands, and the fibrosis envelops individual cells, not nodules of cells. Distinction from diffuse large B-cell lymphoma (DLBCL) can be difficult, and ICC studies are essential to confirm the diagnosis of the reticular subtype. In some cases of LD, features of both diffuse fibrosis and the reticular subtype may be present in different areas of the biopsy specimen.

1.4.5 Lymphocyte-rich classical Hodgkin lymphoma

Cases of LRCHL may resemble mixed cellularity, nodular sclerosis, or nodular lymphocyte predominance HL and may be either nodular or diffuse. Many cases of LRCHL have a close resemblance to MCHL, with a diffuse or vaguely nodular low-magnification appearance. RS cells and variants are relatively rare but, when encountered, have identical features to the RS cells of MCHL. The background is dominated by small mature lymphocytes. Eosinophils and neutrophils are usually restricted to blood vessels. Some cases of LRCHL may show a distinctly nodular appearance that may closely mimic NLPHL. The nodules of LRCHL often contain small reactive germinal centres with HRS cells present in and near the mantle zones, a pattern that has been called follicular HL (Ashton-Key et al. 1995).

1.5 Biology of the Hodgkin and Reed-Sternberg cells of classical Hodgkin lymphoma

The rarity of HRS cells and L&H cells in tissues affected by HL has hampered HL research for decades. Based on morphological and immunological studies, nearly all haemopoietic cells have been postulated as the normal counterpart of HRS cells (Drexler et al. 1989). This list includes lymphocytes, histiocytes, interdigitating dendritic cells, and even granulocytes. With the advent of single cell manipulation and PCR, it is now clear that in more than 98% of cHL cases the neoplastic cells are derived from mature B cells at the germinal centre stage of differentiation (Kanzler et al. 1996; Marafioti et al. 2000). In rare cases, they are derived from peripheral (post-thymic) T cells (Muschen et al. 2000; Seitz et al. 2000).

1.5.1 Immunophenotypic studies of HRS cells

One of the first applications of enzyme labelled ICC was the analysis of HRS cells in formalin-fixed and paraffin-embedded tissue sections for the expression of Ig G (Garvin et al. 1974; Taylor 1974). The HRS cells in all cases studied showed a strong cytoplasmic positivity, pointing to a relationship between HRS cells and IgG-producing B cells. Further studies revealed that the HRS cells not only bound antibodies to IgG but also antibodies to Igκ and λ light chains (Kadin et al. 1978). Because normal B cells produce either Igκ or Ig λ light chains, but never both, it was concluded that the ICC of HRS cells for Igκ and λ represented a technical artefact caused by uptake of Igs from the serum during the fixation process. This observation made it evident that ICC detection of a molecule in a given cell does

not necessarily indicate that it is synthesized by the labelled cell, especially if the molecule under investigation is present in the serum at a significant concentration. Therefore, attempts were made to produce antibodies against cell type-specific molecules that are absent from serum. These attempts succeeded when (a) permanent cell lines derived from HRS cells became available for us as a source of HL-associated antigen and (b) monoclonal antibody technology was established.

1.5.1.1 CD15

The first antigen found to be commonly associated with HRS cells was CD15. This association was first recognized by use of the antibodies Tu9 (Stein et al. 1982) and 3C4 (Schienle et al. 1982), and later Leu-M1 (Pinkus et al. 1985) and C3D1 (Stein et al. 1986). The detection of the CD15 moiety has achieved diagnostic significance because it is present in HRS cells in most cases of cHL but is constantly absent from L&H cells of NLPHL (Pinkus et al. 1985). However, CD15 has no value as a marker of cell lineage because in normal subjects it is expressed on a variety of cells including late cells of granulopoiesis, epithelioid-type macrophages, various epithelial cells, and a subset of B and T cells following activation and/or transformation by EBV (Knapp et al. 1989). It may also be found on HRS-like cells in infectious mononucleosis, and on the neoplastic cells of some NHLs (Wieczorek et al. 1985; Sheibani et al. 1986).

1.5.1.2 CD30

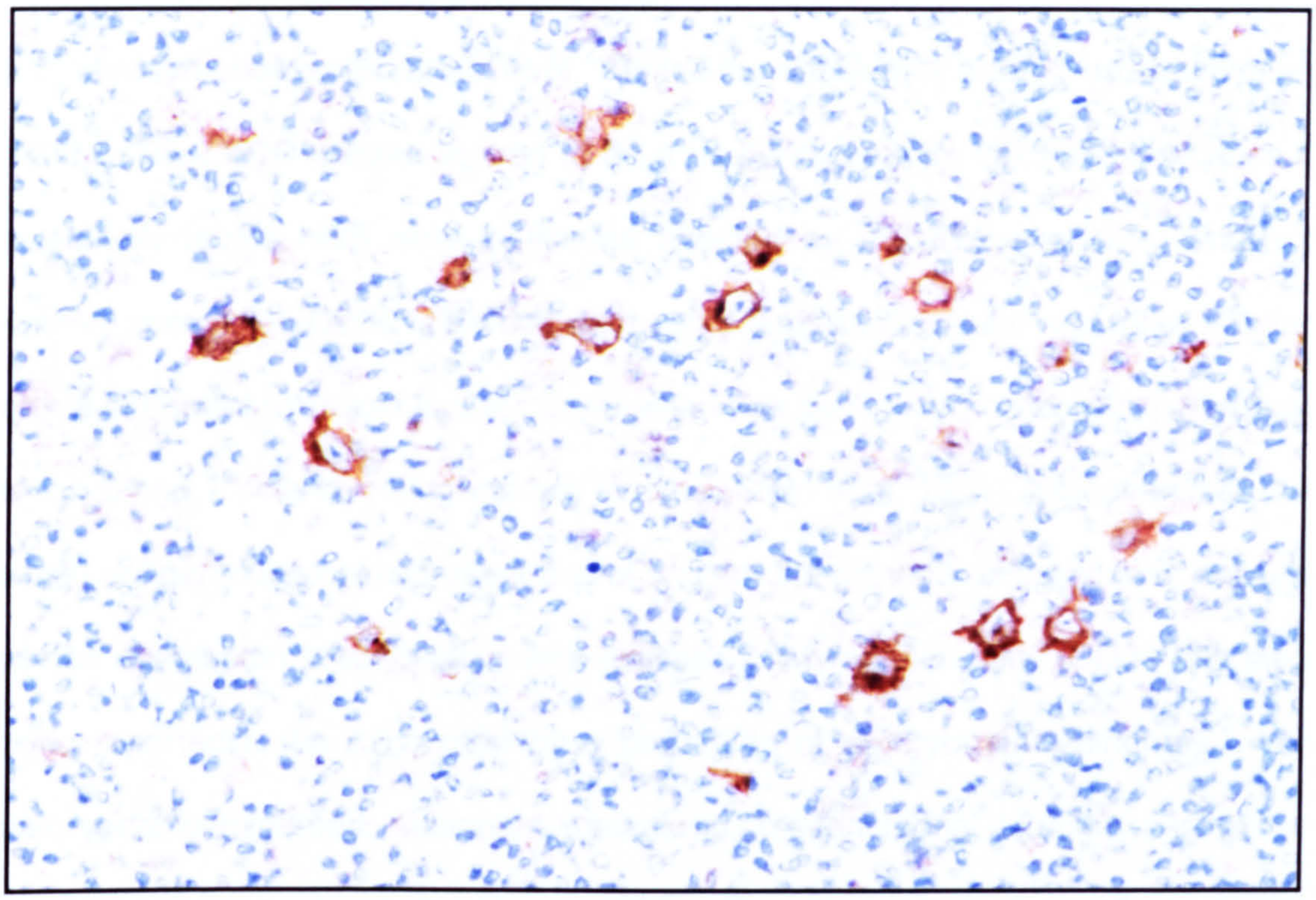

Figure 1.7 Image of CD30-positive HRS cells from a section of paraffin embedded material (x200, DAB counterstained with touludine blue).

In search of viral antigens in HRS cells, in 1981 the research team of Stein generated polyclonal antibodies (Stein et al. 1981), and one year later monoclonal antibodies (Schwab et al. 1982), against the HL-derived cell line L428. These studies led to the discovery of the Ki-1 and Ki-24 molecules (Schwab et al. 1982; Stein et al. 1983), which were subsequently clustered as CD30 (Schwarting et al. 1987) and CD70, respectively (Stein et al. 1989).

By molecular cloning, the CD30 antigen was identified as a cytokine receptor of the tumour necrosis factor receptor (TNFR) family (Durkop et al. 1992). Gene

disruption and functional studies revealed that CD30 is involved in the negative selection of thymocytes and in the regulation of apoptosis and proliferation of activated lymphoid cells (Smith et al. 1993; Shanebeck et al. 1995; Amakawa et al. 1996). The CD30 antigen proved to be a useful marker for HRS cells because it selectively labels HRS cells in tissues affected by cHL (Figure 1.7). It is not entirely specific as CD30 is detected on some large perifollicular and interfollicular blasts in normal lymphoid tissue. Expression can be induced on normal peripheral blood B and T cells by mitogens and viruses (Stein et al. 1985), indicating that the CD30 antigen does not represent a viral antigen but rather a differentiation antigen whose expression is associated with activation of lymphoid cells. CD30 has also been reported to be expressed by a proportion of embryonal carcinomas, non-embryonal carcinomas, malignant melanoma, mesenchymal tumours and some myeloid cell lines (Gruss and Dower 1995). Despite the lack of specificity, CD30 remains the most useful antigen for the identification of HRS cells at the present time.

The overall restriction of the occurrence of the CD30 antigen in normal subjects to occasional activated lymphoid cells strongly favoured a lymphocytic origin for HRS cells (Stein et al. 1985).

1.5.1.3 B- and T-cell markers

In the majority of cases, consistency in the expression of lineage markers in HRS cells is lacking. Supporting the derivation of HRS cells from lymphoid cells was the occasional detection of B-cell antigens (e.g., CD19, CD20, CD22, CD79a) or T-cell

antigens (e.g., CD3, CD4, CD8, TCR β chain) on variable proportions of HRS cells in cHL (Agnarsson and Kadin 1989; Dallenbach and Stein 1989; Schmid et al. 1991a; Zukerberg et al. 1991; Korkolopoulou et al. 1994). Although these findings were generally consistent with the cell lineage characteristics of HL-derived cell lines, they provoked much scepticism. This was because these markers were only demonstrable in a minority of cases, and when present were only present on a proportion of HRS cells. It was therefore widely believed that the detectability of B- and T-cell antigens on HRS cells represented aberrant antigen expression and did not permit any conclusions to be drawn concerning the cellular origin of HRS cells. In contrast, L&H cells were shown to consistently express the B-cell associated molecules J chain (Stein et al. 1986; Schmid et al. 1991b), CD20 (Said 1992), and CD79a (Korkolopoulou et al. 1994), and to lack CD30, CD15 and T-cell antigens (Anagnostopoulos et al. 2000), indicating their distinctness from HRS cells and their derivation from B cells.

More recently, the B cell nature of HRS cells has been further demonstrated by ICC for the B cell specific activator protein (BSAP) in approximately 90% of cases. BSAP is a B cell specific transcription factor and a product of the PAX5 gene (Foss et al. 1999). A further characteristic finding is the absence of the transcription factor Oct2 and / or its co-activator BOB.1. The latter is critical for the induction of Ig transcription in normal B cells (Marafioti et al. 2000).

1.5.1.4 Conclusions from immunophenotypic studies

In summary, these studies agree that the L&H cells of NLPHL consistently express B-cell antigens and so their derivation from B cells is generally accepted. In cHL, the HRS cells in most instances lack lineage-specific antigens and express some unusual molecules, including those characteristic of dendritic cells. However, the antigen profile of HRS cells points towards a derivation from activated lymphoid cells mostly of B-cell type and occasionally of T-cell type.

1.5.2 Cytokines and chemokines

Overexpression and an abnormal pattern of cytokine and chemokine expression is observed in the HRS cells of cHL. They express interleukin (IL) -2 (IL-2), IL-5, IL-6, IL-7, IL-9, IL-10, IL-13 and IL-13 receptor, granulocyte-macrophage colony stimulating factor, lymphotoxin-a, transforming growth factor-β (TGF-β), RANTES, MCP-4, I-309 and eotaxin (Herbst et al. 1996; Kadin and Leibowitz 1999; Teruya-Feldstein et al. 1999; Skinnider et al. 2001). Other interesting but not yet fully understood findings are the expression of restin (Bilbe et al. 1992; Delabie et al. 1992), fascin (Pinkus et al. 1997), and more recently, thymus and activation regulated chemokine (TARC) by HRS cells (Poppema et al. 1999). All three molecules are expressed in normal tissues on dendritic cells. Expression of eotaxin correlates with the extent of eosinophilia within the infiltrate (Teruya-Feldstein et al. 1999). Expression of TGF-β may account for the fibrosis seen in HL. The expression of TARC could contribute to recruitment of T-cells into lesions as well as rosetting of CD4+ T cells by HRS cells.

1.5.3 Ig genes as markers for B-cells and clonality

The clarification of the structure of the Ig gene locus led to the discovery that in B cells – in contrast to all other somatic cells – the Ig genes are rearranged (Rajewsky 1996). This process involves rearrangement of Variable – Diversity – Junction (V-D-J) segments and takes place during the maturation of B cells. The recombination of the Ig gene segments is random and associated with the incorporation of nucleotides (known as N segments) between the rearranged Ig gene segments in the immunoglobulin heavy chain (IgH) gene. A similar process takes place within the immunoglobulin light chain (IgL) genes at a later stage of development. The result is that each single, mature, non-malignant B cell contains distinct IgH and IgL rearrangements that are specific for an individual B cell. In contrast to reactive B cells, neoplastic cells from B-cell lymphoma have identically rearranged Ig genes and thus identical VDJ sequences. Early studies analysing the Ig locus confirmed that each B-cell lymphoma is derived from a single transformed B-cell and so represents – in contrast to the polyclonal proliferation of reactive B cells – a monoclonal B-cell population (Rajewsky et al. 1997).

Analysis of the Ig gene locus in HRS cells and L&H cells therefore presented a method that would unequivocally clarify whether they are B cell-derived and if so, whether they are polyclonal or monoclonal. Many research groups proceeded to analyse DNA extracted from HL biopsy specimens for the presence of clonal Ig rearrangements. Clonal rearrangements were detected in 25% of cases analysed by Gledhill et al in 1991 using Southern blot analysis (Gledhill et al. 1991). The results from other investigators were heterogeneous, with the majority being

negative (Knowles et al. 1986; Roth et al. 1988; Angel et al. 1993). With hindsight this result is not surprising, given that a clonal B-cell population must represent >2-5% of the total cell population before it will be detected by Southern blot analysis of the IgH locus. As previously described, HRS cells or L&H cells rarely exceed 1% of the cell population present in tissue samples affected by HL.

1.5.4 Polymerase chain reaction studies of whole-tissue DNA

The development of the PCR in 1985 (Saiki et al. 1985) and its subsequent simplification (through the use of a thermally stable polymerase) in 1988 (Saiki et al. 1988) opened up a new dimension in the analysis of genes. By means of this method it became possible to amplify specific nucleic acid sequences more than 10^{12} times. Although PCR is exquisitely sensitive and can be used to analyse small samples, this technique does not improve the sensitivity of detection of Ig rearrangements when they are present in a polyclonal B cell background. Therefore PCR did not clarify the cellular origin and the clonality of HRS cells (Tamaru et al. 1994; Manzanal et al. 1995). Where clonal rearrangements were detected, it could not be determined whether they were present in HRS cells or L&H cells or in other cells (e.g. B-cell clones present in a germinal centre).

1.5.5 Polymerase Chain Reaction Studies of Single Cells

The high sensitivity of PCR made it possible to amplify and detect single gene copies for the first time. The emphasis therefore shifted to the isolation of pure populations of HRS cells and L&H cells for molecular biological investigations. In the first study of this kind, single cell cDNA libraries were prepared from putative

HRS cells. They were selected on the basis of their large size, their negativity for CD3, CD14, CD20 and their morphology and were isolated from single cell suspensions by means of a micropipette (Trumper et al. 1993). No consistent pattern of gene expression was detected, casting doubt on the reliability of the HRS cell identification. The research team led by Trumper (Roth et al. 1994) tried to improve cell identification by preparing cytospins from cell suspensions and subsequently ICC for CD30 or CD20 antigens. A second method of HRS cell isolation used thick paraffin sections from which cells were suspended by means of enzymatic digestion and mechanical force (Delabie et al. 1994). The suspended cells were immunostained in solution and then isolated by means of a pipette. A third method, established in the laboratory of Kuppers, isolated CD30+ HRS cells or CD20+ L&H cells from immunostained frozen sections using two hydraulically-driven micromanipulators and micropipettes (Kuppers et al. 1993). The advantage of this method was that the cells to be isolated could be reliably identified on the basis of morphology, antigen expression, and tissue localisation. This technique also avoided the loss of certain cell types, which regularly occurs when tissue is disrupted. However, a high level of manual dexterity was required and the procedure was time-consuming. Furthermore, the use of frozen sections results in nuclear truncation.

1.5.5.1 Preliminary Results and Initial Problems

The first single-cell studies of HRS and L&H cells provided heterogeneous results. Trumper's group failed to demonstrate Ig gene rearrangements in single HRS cells and L&H cells (Roth et al. 1994). In contrast, the research teams of Kuppers

(Kuppers et al. 1994), Chan (Delabie et al. 1994; Delabie et al. 1996; Foss et al. 1999), and Stein (Hummel et al. 1995) found rearranged Ig genes in both HRS cells and L&H cells. Despite this agreement, the results of these groups differed in their rearrangement patterns. Single L&H and HRS cell-derived Ig rearrangements from individual cases reported by Chan's group were unrelated (i.e. polyclonal), whereas those found by Kuppers' group were identical (i.e. monoclonal) (Kuppers et al. 1994). Hummel et al. (1995) observed both rearrangement patterns in HRS cells (i.e., cases with unrelated and cases with identical Ig gene rearrangements). To clarify which one of the observations was valid, Stein's team undertook the following investigations. First, the monoclonal and polyclonal HL cases were reanalysed by using additional primer sets (Stein et al. 1997). This study confirmed the presence of monoclonal Ig rearrangements in the HRS cells of all the cases of cHL already determined to be monoclonal. However, in two of the four cases with polyclonal rearrangements, the reinvestigation led to the detection of identical (monoclonal) rearrangements, showing that the monoclonal rearrangements had escaped detection with the consensus primers used in the first analysis. Secondly, to understand why polyclonal Ig rearrangements were originally detected in some cases, rearrangement patterns were correlated with the cellular composition of the sections used for the isolation procedure. This analysis revealed that all of the sections that gave rise to polyclonal rearrangements in the single-cell assay contained a significant number of reactive B cells, whereas reactive B cells were rare or missing in the cases without polyclonal rearrangements. This strongly

suggested that the polyclonal rearrangements stemmed from the contaminating DNA of reactive small B cells rather than from HRS cells. The subsequent examination of the cell isolation procedure revealed that this was indeed the case. It was shown that the DNA of reactive B cells tends to float off into the buffer covering the frozen sections during cell isolation; there was therefore a great risk of including contaminating B cell-derived DNA in the PCR assay when the selected HRS cell or L&H cell was transferred to the PCR tube with a large volume of covering buffer (Marafioti et al. 1997).

1.5.5.2 Final Results

With optimisation of the cell isolation procedure, and the use of new sets of IgH gene and IgL gene primers, Stein's group investigated 1078 single Hodgkin and Reed-Sternberg (HRS) cells and 615 single L&H cells from 25 cHL cases (Marafioti et al. 2000) and 11 NLPHL cases (Marafioti et al. 1997). All analysable cells of all NLPHL cases and 24 of the 25 cHL cases showed identical Ig gene rearrangements in a given case. An unrelated rearrangement was found in only one out of 1639 cells. These findings confirmed that the HRS cells and the L&H cells of most cHL cases and all NLPHL cases contain monoclonal Ig gene rearrangements. These results also vindicated the results of Kuppers et al. (1994), who had arrived at the correct result in their initial analysis.

In follow-up studies, 15 additional cases of cHL and 5 additional cases of NLPHL were analysed (Kanzler et al. 1996; Braeuninger et al. 1997). In all but one of the cases clonal Ig gene rearrangements were detected. In two of the cases CD30+

cells were enriched by magnetic activated cell sorting from the lymph node biopsy. It was demonstrated by Ig gene analysis that the enriched cells indeed represented the primary HRS cells of the patient. This showed that viable HRS cells represent clonal populations throughout the involved lymph nodes (Irsch et al. 1998). In addition, the question of dissemination and persistence of the HRS cells in the patient during the course of the disease was addressed. In each of 3 cases, it was shown that the same HRS tumour clone could be found in different lymphoid organs and that relapses of the disease were caused by the original lymphoma clone. Dissemination of lymphoma cells was also observed in two cases of NLPHL (Jox et al. 1998).

In conclusion, the demonstration of Ig gene rearrangements in the HRS cells in approximately 98% of cases of cHL and in L&H cells in all cases of NLPHL proved the B-cell nature of these cells in most or all instances, respectively. The presence of monoclonal Ig gene rearrangements in HRS cells and L&H cells indicates their origin from a single transformed B cell and a subsequent monoclonal expansion. The failure to detect rearranged Ig genes in HRS cells and L&H cells was a false-negative finding.

1.5.6 Mapping of HRS cells and L&H cells onto normal B-cell development

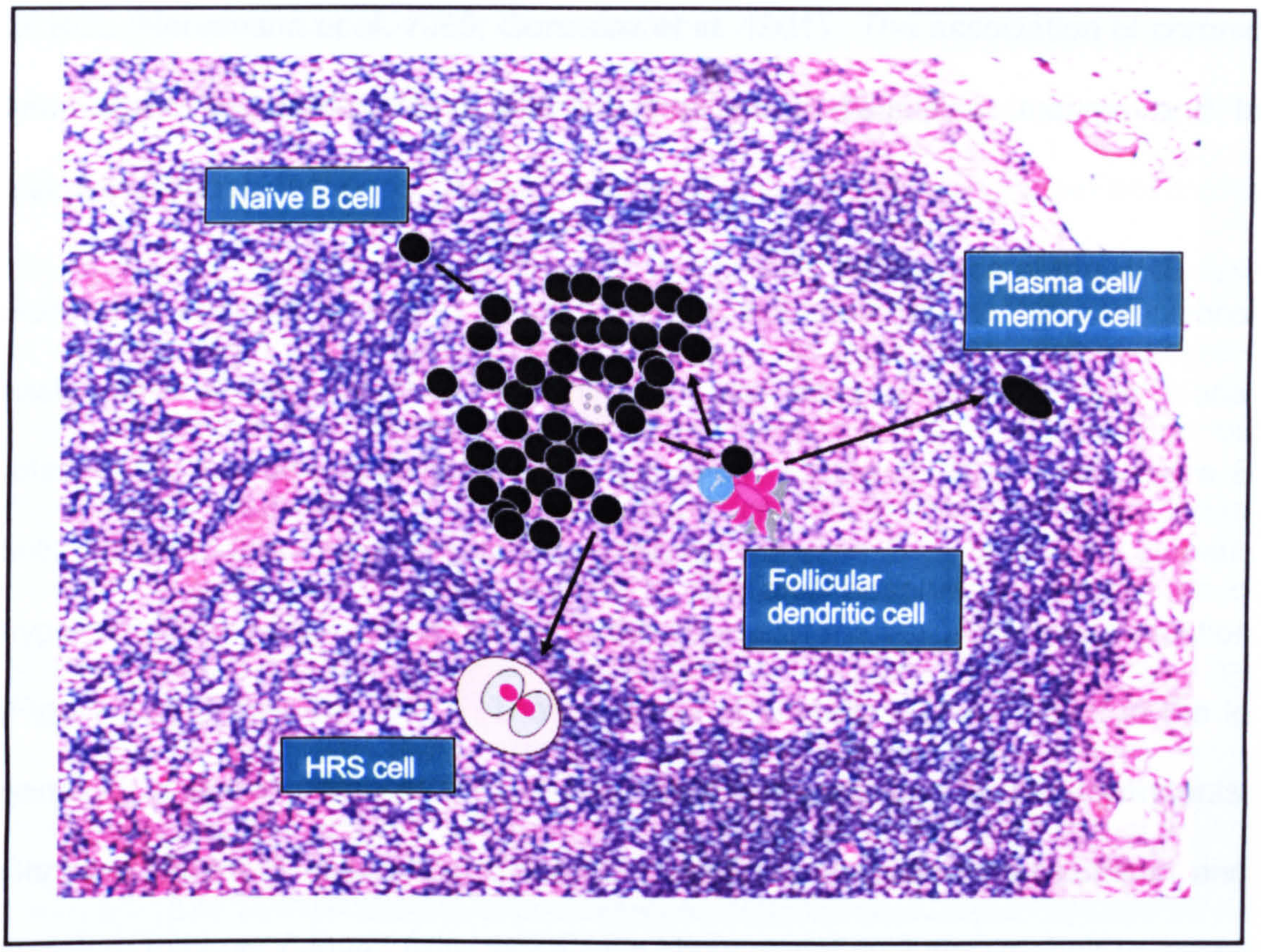

Figure 1.8 Diagram of germinal centre B cell development. Naïve B cells, which have matured in the marrow, enter the germinal centre of lymph nodes following exposure to antigens and activation. Somatic hypermutation will take place here and only B cells with high affinity will be selected to become either a plasma cell or memory cell. Most of the B cells are eliminated by apoptosis. Hodgkin and Reed-Sternberg (HRS) cells are thought to derive from pre-apoptotic germinal centre B cells.

Composite lymphoma may be defined as the simultaneous occurrence of HL and NHL in the same anatomic site or biopsy specimen (Kim et al. 1977). The most common form is B-cell NHL occurring in association with cHL, usually of the NS or MC subtype. The type of B-cell NHL involved reflects the incidence of B-cell

lymphoma subtypes in the population. Most composite lymphomas involve FL and DLBCL (Hansmann et al. 1989; Gonzalez et al. 1991). The association of chronic lymphocytic leukaemia (CLL) with HL has some distinctive features and is discussed below.

Sequence analysis of the Ig rearrangements not only allows the unequivocal assignment of cells to the B-cell lineage and determination of their clonal relationship, but also specifies their stage of development. Mature but naive B cells harbour rearranged IgH genes, whereas activated B cells acquire somatic hypermutations within the IgH genes in the course of the germinal centre reaction (Figure 1.8) (Rajewsky 1996). The presence of somatic hypermutation in the Ig genes is a specific marker for germinal centre B cells and their descendents. Germinal centre B cells show evidence of ongoing somatic hypermutation and therefore tumours derived from these cells show clonal evolution of their Ig genes (Klein et al. 1998). Comparison of the sequences of HRS cell-derived Ig gene rearrangements with corresponding germ-line segments demonstrated high loads of somatic mutations in all instances. In some cases these were associated with the presence of stop codons and deletions (termed 'crippled' mutations) (Kanzler et al. 1996; Marafioti et al. 2000). Although these findings indicated that HRS cells of cHL display some molecular features of germinal centre B cells, they did not determine the precise relationship between HRS cells and germinal centre B cells. This gap was closed in an investigation of Ig gene rearrangements and their mutation patterns in three cases of composite lymphoma (Brauninger et al. 1999; Marafioti et al. 1999).

In the 3 cases studied, the HRS cells and the NHL B-cells of the composite lymphoma contained identical Ig gene rearrangements, indicating their derivation from the same B-cell precursor. All rearrangements carried somatic mutations; some of these were shared by the HRS cells and the NHL cells, whereas others were exclusive to each cell type. The rearrangements in the 3 NHLs, but not those in the HRS cells, showed signs of ongoing mutation, i.e. individual clonally-derived cells had different mutations. The shared sequences unequivocally mapped the differentiation stage of the identified common B-cell precursor as that of a germinal centre B cell and ruled out the progression of HRS cells to NHL cells. These findings allowed the following conclusions to be made. First, HRS cells are derived from germinal centre B cells and not from post-germinal centre B cells. Secondly, the descendants of the common B-cell precursor can undergo two separate transforming events, one leading to HRS cells and the other to NHL. Thirdly, the transforming event producing HL totally changes the morphology and immunophenotype of the common precursor cell, while the NHL transforming event more or less maintains the features of the precursor B cell.

The development of cHL in a patient with CLL has been recognised for some time and has been considered a form of Richter's transformation (Choi and Keller 1981). Ohno et al. (1998) attempted to examine the clonal relationship between CLL and HL using microdissection techniques and PCR amplification of IgH genes. The IgH gene sequences from the HRS cells were identical to those from the CLL cells in two cases. These studies provide further evidence that the HRS cells in both CLL and cHL may share the same mature B-cell precursor. However,

in this study the differentiation stage of the common precursor cell could not be assessed because the analysis was restricted to the CDR3 of the rearranged Ig gene only. A case report on a composite mantle cell lymphoma (MCL) and cHL demonstrated separate clonal origins from the tumour cells (Caleo et al. 2003).

NLPHL is also derived from a B cell, as indicated by clonal IgH rearrangements of the L&H cells (Braeuninger et al. 1997; Marafioti et al. 1997; Ohno et al. 1997). The V-D-J regions of L&H cells show intraclonal diversity indicating continuation of somatic hypermutation after the initial transforming event. Most of the analysable sequences from L&H cells are functional suggesting that these cells have been positively selected by antigen (Kanzler et al. 1996). L&H cells express B cell lymphoma / leukaemia (BCL)-6, a transcriptional repressor protein characteristically expressed in germinal centre B cells (Staudt et al. 1999). This is in marked contrast to the HRS cells in cHL which do not show evidence of continuing somatic hypermutation, have 'crippling mutations' (Kuppers and Rajewsky 1998) and usually lack BCL-6 expression (Carbone et al. 1998). In contrast, the HRS cells in cHL appear to undergo a transformation event early in their evolution that makes cell survival independent of antigen receptor signaling. L&H cells appear to behave like centroblasts, with continued somatic hypermutations in a germinal centre-like environment, and this may be important for persistence and expansion of the clonal population (Chan 1999).

1.5.7 T-cell type of classical Hodgkin lymphoma

Repeated reports of the detection of T-cell antigens in HRS cells, in conjunction with the establishment of HL-derived cell lines having immunophenotypic and genotypic features of T cells, pointed towards the existence of T cell-derived HRS cells (Cibull et al. 1989; Dallenbach and Stein 1989). Early studies investigating whole tissue DNA failed to detect TCR rearrangements in HL (Gledhill et al. 1990). To investigate this possibility further, immunophenotypical studies were extended to include the cytotoxic molecules perforin and granzyme B, because these were found to be specific markers for cytotoxic T cells and natural killer cells (Oudejans et al. 1996). These studies showed that HRS cells of 10% to 20% of cases of cHL express one or both cytotoxic molecules, and that the presence of these molecules is often associated with expression of the T-cell markers CD3, CD4, CD8 and/or the TCR β chain. Although these findings suggested the existence of T-cell-derived HRS cells, they were not regarded as conclusive. Later genotyping studies showed that only a minority of cases expressing T-cell markers had TCR rearrangements, indicating that the T-cell antigens expressed by HRS cells are not lineage specific (Muschen et al. 2000; Seitz et al. 2000).

1.6 Epidemiology of Hodgkin lymphoma

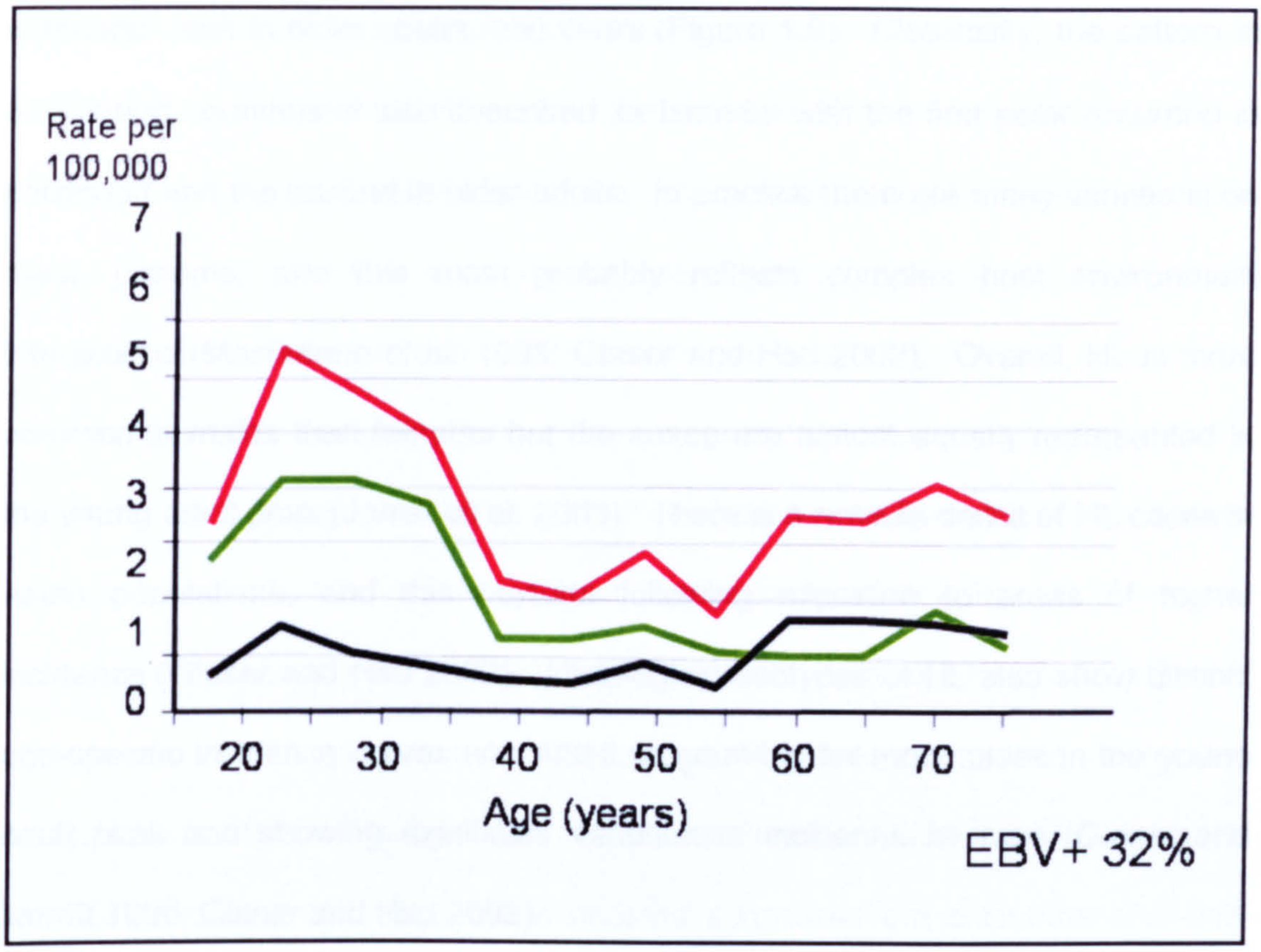

Figure 1.9 Incidence of cHL in Scotland (Jarrett et al. 2003) showing bimodality with a young adult peak and a smaller peak in older adults. Red line is overall incidence. Green line is incidence for non-EBV associated cases. Black line is EBV associated cases. Overall, 32% of all cHL in Scotland are associated with EBV.

The epidemiology of HL is characterised by features that are unusual for a malignancy and suggest both aetiological heterogeneity and involvement of infectious agents (Glaser and Jarrett 1996). The international variation in incidence is relatively small compared to some cancers but incidence patterns manifest substantial variation with respect to age, sex, ethnicity and histological subtype. In developed countries, HL is characterised by a bimodal distribution with

a first age-specific peak in young adults between the ages of 15 and 34 years and a second peak in older adults, ≥50 years (Figure 1.9). Classically, the pattern in developing countries is also described as bimodal with the first peak occurring in childhood and the second in older adults. In practice there are many variations on these patterns, and this most probably reflects complex host environment interactions (Macfarlane et al. 1995; Glaser and Hsu 2002). Overall, HL is more common in males than females but the sexes are almost equally represented in the young adult peak (Jarrett et al. 2003). There is a notable deficit of HL cases in Asian populations, and this persists following migration to areas of higher incidence (Glaser and Hsu 2002). Histological subtypes of HL also show distinct age-specific incidence curves with NSHL accounting for most cases in the young adult peak and showing significant variation in incidence by race (Glaser and Jarrett 1996; Glaser and Hsu 2002).

1.6.1 Hypotheses of aetiology

Consideration of many of the above features led MacMahon, almost 50 years ago, to suggest that HL was not a single disease entity (MacMahon 1966). He suggested that HL occurring in children, young adults and older adults was likely to have a different aetiology – the multiple aetiology hypothesis. Furthermore, he put forward the idea that HL in young adults was caused by an infectious agent. Many subsequent studies have shown that risk factors for disease development are different in these age groups. In particular, young adult HL is associated with risk factors suggesting a high standard of living and social isolation in childhood

(Gutensohn and Cole 1981). It has been inferred from these data that young adult HL is caused by delayed exposure to a common infectious agent – the delayed exposure hypothesis or late host response model. Consistent with this, young adult HL cases report fewer childhood infections than age-matched controls (Alexander et al. 2000). A seminal paper by Correa and O'Conor (1971) reported that rates of childhood and young adult HL were inversely proportional (Correa and O'Conor 1971). This study influenced much thinking about HL aetiology over the next two decades; in particular it led to comparisons between HL and paralytic polio in the pre-vaccine era – the polio model. This suggested that HL in children and young adults was caused by the same infectious agent but that age of infection influenced the likelihood of disease. A later study by MacFarlane and colleagues extended and reanalysed these data but concluded that this inverse relationship was no longer apparent and perhaps never existed (Macfarlane et al. 1995). The latter data are more in keeping with the multiple aetiology hypothesis.

1.6.2 Epstein-Barr virus in classical Hodgkin lymphoma

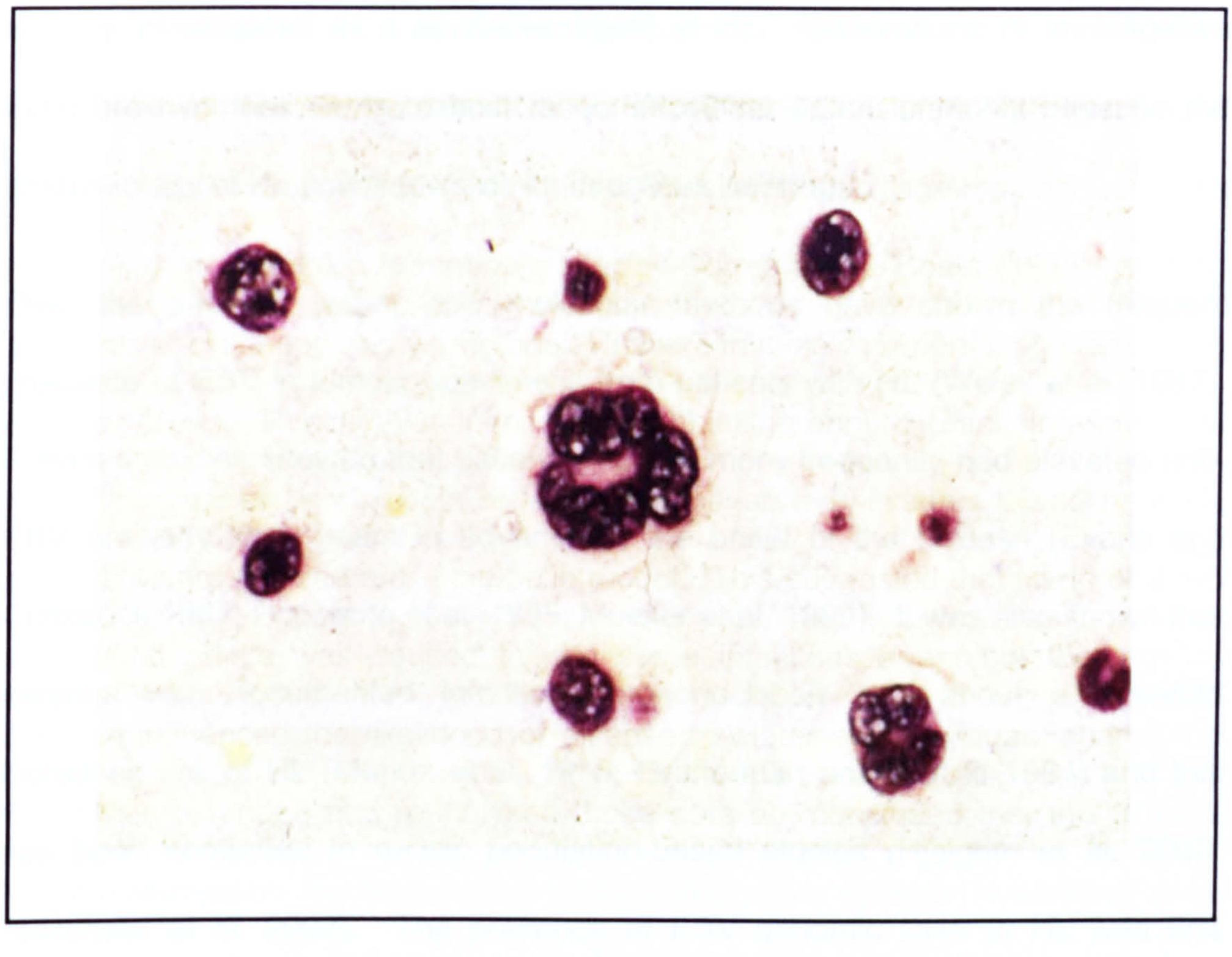

Figure 1.10 Image of EBER *in situ* hybridisation in a case of EBV-positive cHL. Positive staining is localised within the nuclei of HRS cells (x400).

The family herpesviridae comprises over 100 viruses with similar biochemical and morphological characteristics. Herpesviruses are highly disseminated in nature and have been isolated from many animal species. A property of herpesviruses is their ability to remain in a latent state within the host following primary infection. The site of latency varies between herpesviruses, but includes lymphocytes, monocytes and neuronal tissue. The Epstein-Barr virus (EBV), first isolated from a Burkitt's lymphoma (BL) specimen, can be classified as a gamma-one herpesvirus because of its B cell lymphotropism or as a group C herpesvirus because of its

genome sequence arrangement (Roizman and Pellett 2001). EBV has been actively investigated as a causative agent in HL. Generations of investigators have believed that HL is caused by an infectious agent, primarily because the epidemiology of HL points towards an infectious aetiology.

Over the past 15 years, new molecular methods have shown the frequent presence of EBV in tumour specimens from patients with HL (Weiss et al. 1987). Previous studies showed that patients with HL more frequently had elevated anti-EBV antibody titres years in advance of the onset of the disease (Evans and Comstock 1981; Poppema et al. 1985; Mueller et al. 1989). It was also shown that persons with documented infectious mononucleosis had about a threefold increased risk of HL (Munoz et al. 1978; Grufferman and Delzell 1984) and this has been confirmed in recent population-based studies (Hjalgrim et al. 2000; Alexander et al. 2003). The presence of EBV genomic DNA in HL was first reported by dot blot and Southern blot hybridisation studies in 1987 (Weiss et al. 1987) and was soon confirmed in many other studies (Anagnostopoulos et al. 1989; Staal et al. 1989; Bignon et al. 1990; Gledhill et al. 1991). By means of these techniques, about 20% to 30% of cases of HL were shown to contain substantial levels of EBV genomes. Using a probe directed against DNA sequences adjacent to the EBV terminal repeats, the EBV genome was shown to be clonal in most cases, indicating clonal expansion of a single EBV-infected cell (Weiss et al. 1987; Gledhill et al. 1991).

Although the above studies indicated that EBV genomes were present in HL, it was essential to demonstrate the cellular localisation of the EBV. Pallesen et al. (1991) and Herbst et al. (1991) used monoclonal antibody frozen section ICC to identify abundant expression of EBV latent membrane protein (LMP) 1 in HRS cells in approximately 50% of cases of HL. Subsequent studies, particular those using modern ICC techniques, have confirmed their findings (Brousset et al. 1994). *In situ* hybridisation studies, most often utilizing probes to Epstein-Barr viral polymerase III transcript (EBER) RNA, also localised the virus to HRS cells (Wu et al. 1990; Pallesen et al. 1991; Khan et al. 1992). Studies have shown that LMP1 paraffin-based ICC studies and EBER *in situ* hybridisation yield nearly identical results in HRS cells (Hamilton-Dutoit et al. 1991; Jarrett 1992). In any given EBV-positive case, approximately 50% to 90% of the HRS cells are usually LMP1 positive whereas all, or almost all are EBER positive. In contrast to EBER probes, LMP1 antibodies do not stain small lymphocytes. EBER is a more robust technique but LMP1 is more specific. These techniques conclusively showed that EBV genomes are present in the HRS cells. In addition, all HRS cells within a lesion, or within a case, of EBV-associated HL were shown to contain EBV.

ICC studies further showed that the LMP1 protein was expressed by HRS cells. Epstein-Barr viral nuclear antigen (EBNA) 1 (Grasser et al. 1994) and LMP2 (Niedobitek et al. 1997) are also expressed but EBNA2 is consistently lacking, a type of EBV latent infection that is referred to as latency II (Deacon et al. 1993). LMP1 and possibly LMP2 are likely to play some role in HRS cell survival (Babcock and Thorley-Lawson 2000). On the basis of the above data, the

International Agency of Research on Cancer (IARC) consensus is that EBV does play a role in the pathogenesis in EBV associated cHL (see section 1.6).

1.6.3 EBV and the epidemiology of HL

EBV positivity appears to correlate with geographical, cultural, genetic, and/or socio-economic influences, all of which are difficult to separate. Among persons from the United States, most parts of Europe, and Israel, approximately 40% to 50% of cases of HL have been shown to contain EBV-positive HRS cells (Glaser et al. 1997). In Scotland, 33% of cHL are EBV-associated (Jarrett et al. 2003). In contrast, among populations from less developed regions, particularly those with a large number of paediatric cases of HL, a very high frequency of EBV has been found. One study found an EBV frequency of 94% in the HRS cells of childhood cases of HL among an indigenous Indian population from an underdeveloped area of Peru (Chang et al. 1993). Ethnicity influences EBV-positivity rates, with higher proportions of EBV-positive cases among Hispanics (Glaser et al. 1997).

Cases of MCHL are more likely to be EBV-associated than NSHL cases but overall more NSHL than MCHL cases are EBV-associated. Males outnumber females (approximately 2:1) among EBV-associated cases but in the non-EBV-associated cases the males and females are almost equally represented (Jarrett et al. 2003). EBV-associated cases are also relatively more common in children and older adults compared to young adults (Jarrett 2002).

Two population-based epidemiological studies aimed at investigating risk factors for development of HL with cases stratified by EBV status were performed by our group – a study of HL in young adults and the Scotland and Newcastle Epidemiological study of Hodgkin Disease (SNEHD) (Alexander et al. 2000; Alexander et al. 2003; Jarrett et al. 2003). The age-specific incidence of EBV-associated and non-associated cases analysed separately were determined using the SNEHD data (Jarrett et al. 2003). These two 'entities' show distinct incidence curves with non-EBV-associated cases accounting for the young adult peak and showing a unimodal age-specific incidence curve. EBV associated cases appear to have a bimodal incidence curve with a small peak in the 15-24 year age range and a larger peak in the older adult age group.

The association between previous history of infectious mononucleosis (IM) was analysed in both studies. In the young adult study, the increased risk associated with IM was found to be specific for EBV-associated HL (Alexander et al. 2000). In SNEHD, cases were more likely than controls to have had previous IM and case-control differences were significant for both EBV-associated and non-associated cases (Alexander et al. 2003). However, risk was greatest for young adult EBV-associated cases and the time from IM to the diagnosis of HL was also shorter. Hjalgrim et al. (2003) examined incidence of HL in cohorts of subjects who had been serologically tested for acute EBV infection; positive results were associated with an increased risk of developing EBV-associated but not non-EBV-associated HL. Taken together, these studies provide robust evidence for a causal link between IM and EBV-associated HL in young adults. Cases occurring following

IM, and therefore associated with delayed exposure to EBV, most probably account for the first peak in the age-specific incidence of EBV-associated HL.

1.6.4 EBV-negative cHL

Although the precise function of EBV in cHL is not known, there is now substantial data supporting the notion that EBV is playing a causal role in cHL. Of those HL cases that are EBV negative, their aetiology remains obscure. Jarrett et al. (1996) have proposed that infection with another oncogenic virus may be responsible for EBV-negative HL. Extensive searches have been performed looking for other known viruses, including human herpes virus (HHV) 6, HHV7, HHV8, herpes simplex virus, varicella zoster virus, cytomeglovirus, measles, rubella, adenovirus, papovavirus, adenovirus, lymphotrophic papovavirus, SV40, and human T-cell leukaemia virus (Jarrett and Armstrong 1995); to date, all of these studies have given rise to inconsistent or negative results. It is therefore suspected that another novel infectious agent may be involved (Gallagher et al. 2002). An alternative hypothesis is that the EBV is involved in the aetiology of essentially all of HL cases, but the viral genome itself is somehow lost from the HRS cells (hit and run) in patients with a stronger host response (Ambinder et al. 1993). There is currently no evidence to support hit-and-run mechanism in cases classified as non-EBV-associated (Mueller 1997; Gallagher et al. 2003).

1.6.5 Four disease model

The above data led to the proposal of a four disease model in HL (Jarrett 2002). This suggests that there are three EBV-associated diseases: a childhood disease

accounting for most cases of HL below the age of 10 years; a young adult disease associated with delayed exposure to EBV; and an older adult disease, most probably related to reactivation of EBV. Superimposed on these is a non-EBV-associated disease, which accounts for most cases in young adults in developed countries. Epidemiological evidence suggests that these cases are also linked to an infectious agent but no specific agent has been identified to date. The absolute incidence of each of these diseases will be determined by both environmental and host factors and the relative magnitude of each of the diseases will determine the overall shape of the age-specific incidence curve in any particular setting. The model provides a framework to study the heterogeneity of HL in future epidemiological and laboratory studies.

1.7 Genetic, environmental and non-viral factors

There are many early reports describing the occurrence of multiple cases of HL in families (Barretto et al. 1984). In a population-based study, Grufferman et al. (1977) found an increased HL risk in siblings of young adult patients (sevenfold) but no increased risk in siblings of older adults with the disease. Siblings of the same sex had twice the risk of developing HL compared to siblings of the opposite sex. This was thought to be due to sex-concordant sibling pairs having more shared environmental exposures, which was confirmed in a later study (Grufferman et al. 1987). More recently, Mack et al (1995) reported a remarkably increased risk in monozygotic twins of HL cases. They reported that 10 twins of 179 identical twins with HL also had the disease, compared to none of 187 dizygotic twins with HL. The relative risk was 99 times. This observation provided

a strong argument in favour of genetic susceptibility although shared intrauterine exposure is another explanation for these findings.

A case-control study of childhood HL suggested that first-degree relatives of patients younger than 15 years of age at diagnosis had a 2.7-fold increased risk of all cancers (Grufferman et al. 1998). In a series of 464 HL cases and 699 matched controls, 29 cases and 17 controls had a first-degree relative with a diagnosis of cancer. Four cases but no controls had parents with HL (Grufferman et al. 1998). Treated cHL patients have an increased risk of developing second cancers (Strom et al. 1997; Strom et al. 1998). High levels of pre-treatment sister chromatid exchanges (SCE) and age are predictors of second cancer risk. Histology, stage and treatment are not associated with elevated risk (Strom et al. 1998).

An analysis of 41 multiplex families provided strong evidence of linkage between a susceptibility gene tightly linked to the HLA locus and HL (Chakravarti et al. 1986). This putative locus could potentially account for 60% of familial cases. The remaining 40% of cases are more likely to be due to other familial/polygenic or environmental factors. The role played by HLA genes in the control of immune responses to viruses suggests that HLA genes might be important in the aetiology of cHL (Alexander et al. 2001).

1.8 Permanent Hodgkin lymphoma-derived cell lines

Attempts were made to grow permanent cell lines from HL samples in order to circumvent some of the problems associated with analysis of primary tumour

material. The first two permanent cell lines (designated L428 and L540) that were likely to be derived from HRS cells were established in Diehl's laboratory in 1979 (Schaadt et al. 1979). A total of fifteen HL-derived cell lines have now been established. Most of the cell lines have been grown from body fluids (bone marrow (BM), pleural effusion, peripheral blood) of patients with advanced-stage disease. Despite the close resemblance to HRS cells in immunophenotype, it is not generally accepted that all of these HL-derived cell lines are truly derived from HRS cells. This is because the HL-derived cell lines are not homogeneous in that two-thirds of them exhibit the immunophenotypic and genotypic features of B cells, and the other third the characteristics of T cells. In addition the karyotypes of the cell lines are grossly disordered without having consistent cytogenetic aberrations and among the 15 HL-derived cell lines only one (L591) is EBV-positive, whereas *in vivo* the HRS cells of cHL are EBER positive in 30-50% of cases (Herbst et al. 1992; Hummel et al. 1992; Jarrett et al. 2003). There are 2 explanations for this bias; first, the EBV might have been lost during establishment of the cell line and secondly, many true EBV-positive HL-derived cell lines might have been discarded because they were thought to be EBV immortalised lymphoblastoid cell lines (LCLs), which also express the CD30 antigen.

Only one of the above-mentioned HL-derived cell lines has a proven derivation from HRS cells. For the L1236 cell line, established in 1996, it was shown that the cells harbour the same Ig gene rearrangements as the *in situ* HRS cells from the patient from whom the cell line was established (Wolf et al. 1996).

HL-derived cell lines provide an important reagent for research into HL. The cell lines were used successfully for: the discovery of HRS cell-associated antigens including CD30 (Schwab et al. 1982), CD70 and Ki-27 (Stein et al. 1983); for cloning the CD30 gene; and for studying the CD30 signal transduction pathway. Moreover, they have enabled the *in vitro* testing of new immunotherapeutic modalities such as CD30-linked agents (Engert et al. 1990; Falini et al. 1992; Hombach et al. 1993; Pohl et al. 1993). However, it is quite clear that these lines are probably not representative of HRS cells in early stage disease.

1.9 Animal models for Hodgkin lymphoma

There is no good natural or experimental animal model for HL. Nude mice are a mutant strain of bald mice that congenitally contain very little thymus tissue and so are incapable of mounting the strong T cell response involved in rejecting tumour cells. Injecting tumour cells into nude mice has not allowed successful propagation of HL (von Kalle et al. 1992). Severe combined immunodeficient (SCID) mice lacking functional B and T lymphocytes were described in 1983 (Bosma et al. 1983) and seemed to be a better alternative to nude mice, allowing the successful propagation of human lymphoma cells and even human untransformed B cells. SCID mice were tested as a potential animal model for HL. In the first instance, HL-derived cell lines were xenotransplanted subcutaneously. In contrast to the situation in nude mice, all cell lines tested (L428, L540, L591, HD-LM2, KM-H2) did grow progressively and reproducibly after subcutaneous inoculation into SCID mice without prior treatment of the animals (von Kalle et al. 1992). Based on these observations, HL-derived cell lines were also inoculated

intravenously into the tail veins of SCID mice to achieve disseminated growth resembling the growth pattern of HL in humans. When this approach was used, of three cell lines tested (L540, L428, and KM-H2) only the L540 cell line gave rise to tumours (Kapp et al. 1994). After intravenous inoculation, L540 cells showed progressive disseminated growth and preferentially localized in lymph nodes, particularly the cervical, iliac, and inguinal nodes. The spleen was rarely involved. This disseminated SCID mouse model for the growth of HL cells was successfully used in the preclinical *in vivo* testing of new immunotherapeutic modalities (Winkler et al. 1994). In the meantime, further HL-derived cell lines showed disseminated growth in SCID mice. These observations encouraged the transplantation of primary HRS cells into this mouse strain (Kapp et al. 1993). Lymph node or spleen tissue affected by HL was transplanted into the subrenal capsule of SCID mice. Only the material of three patients, from a total of 13 tumours of human origin, developed and spread predominantly into the lymph nodes. However, the tumours were not derived from HRS cells; an outgrowth of EBV-positive B lymphocytes similar to lymphoblastoid cell lines (LCL) was observed. They were found to be clonally distinct from HRS cells by IgH rearrangement. These LCL-like B cells displayed a high number of numeric and structural chromosomal aberrations when compared with EBV-positive B cells growing out from SCID mice after transplantation of B cells from healthy donors. This observation suggests an inherent cytogenetic instability of the B lymphocytes surrounding HRS cells. A similar conclusion was reached in a study from our laboratories and at present this

model is of limited usefulness for studying the biology of HL (Krajewski et al. 1995).

In conclusion, HL transplanted into SCID mice has not yet been proven to be a satisfactory model for the study of HL as there is currently no evidence that HL-derived SCID tumours are related to the neoplastic cells in the original tumour (Krajewski et al. 1995).

1.10 Oncogenes and tumour suppressor genes in classical Hodgkin lymphoma

The scarcity of HRS cells in tissue affected by HL has hampered not only the elucidation of their lineage and origin but also the detection of dysregulated expression of specific oncogenes and inactivation of tumour suppressor genes. Analysis of HL-derived cell lines has not revealed consistent patterns of oncogene expression (Durkop et al. 1992). Since nearly all of these cell lines were established from end-stage HL, genetic alterations present in these cell cultures might be related to the use of mutagenic agents in the course of therapy. When tissue sections of cHL were analysed for genomic alterations or deregulated expression of oncogenes such as *myc*, *jun*, *raf*, and *ras*, no characteristic abnormal pattern could be detected (Steenvoorden et al. 1988). The same holds true for the investigation of tumour suppressor genes (Piris et al. 1995).

The retinoblastoma (Rb) tumour suppressor gene, which is involved in cell cycle regulation, is mutated and/or deleted on both alleles in many malignancies, resulting in the absence of RNA and protein. In most of the HL cases analysed, expression of the Rb protein was detected (Weiss 1995; Sanchez-Beato et al.

1996a). The p53 tumour suppressor gene is also found to be expressed in most cases of cHL (Piris et al. 1995). The unmutated p53 gene codes for a protein involved in control of the cell cycle and induction of apoptosis after DNA damage. Nuclear accumulation of p53 is frequently observed in HRS cells. Mutations in the p53 gene have been detected in only a subset of cases of HL including one cell line (Gupta et al. 1993; Maggio et al. 2001). Thus, the significance of detection of p53 protein in HRS cells remains unclear at present. Overexpression of p53 is usually associated with mutation but it might also be caused by inactivation of p53-binding proteins. The protein encoded by the mouse double minute (MDM) 2 oncogene can bind p53. Overexpression of the MDM2 gene product simultaneously with overexpression of p53 has been described in HRS cells (Chilosi et al. 1994). Similarly, other gene products interacting with p53 and involved in the regulation of the cell cycle are expressed in a proportion of HRS cells and L&H cells of most HL cases suggesting a preservation of p53 functionality (Sanchez-Beato et al. 1996b).

In the search for recurrent chromosomal alteration in HRS cells, many PCR studies have looked for t(14:18) chromosome translocations. This translocation results in dysregulated expression of BCL-2, which can prevent apoptotic death of tumour cells. Whereas some studies have not found any evidence of t(14:18) in HL (Weiss 1995), others have detected this translocation in a variable proportion (6-39%) of cases (Bhagat et al. 1993; Weiss 1995). In positive cases, it remained unproven whether the translocation was localized in the HRS cells, particularly because detection of the BCL-2 protein by ICC *in situ* was not congruent with

detection of the translocation itself (Bhagat et al. 1993). In a more recent study investigating isolated single HRS cells for the presence of t(14:18), it was shown that this translocation was localized in non-malignant bystander B cells and not in HRS cells (Gravel et al. 1998). Similarly, no pathogenetic role in HL could be established for the chromosomal translocation t(2:5), which results in the oncogenic nucleophosmin (NPM) / anaplastic lymphoma kinase (ALK) fusion transcript and is a characteristic of ALK positive anaplastic large cell lymphoma (ALCL) (Trumper et al. 1997).

1.11 Mechanisms preventing apoptosis

The lymphoid immune system exists in a state of homeostasis, with extensive and rapid clonal expansion matched by massive cell death in almost all phases of development. Death by apoptosis occurs in the vast majority of lymphocytes and lymphoid progenitors. The apoptotic pathway is critical in the attenuation of immune responses and in the elimination of autoreactive cells. Deregulation of apoptosis has been associated with immunodeficiency states, lymphoma and autoimmune disease (Rudin and Thompson 1998). Germinal centre B cells with crippled antigen receptors regularly arise in the germinal centre; such cells are usually eliminated efficiently by apoptosis and are not allowed to leave the germinal centre microenvironment (Weiss et al. 1992; Rajewsky et al. 1997).

1.11.1 NFκB family

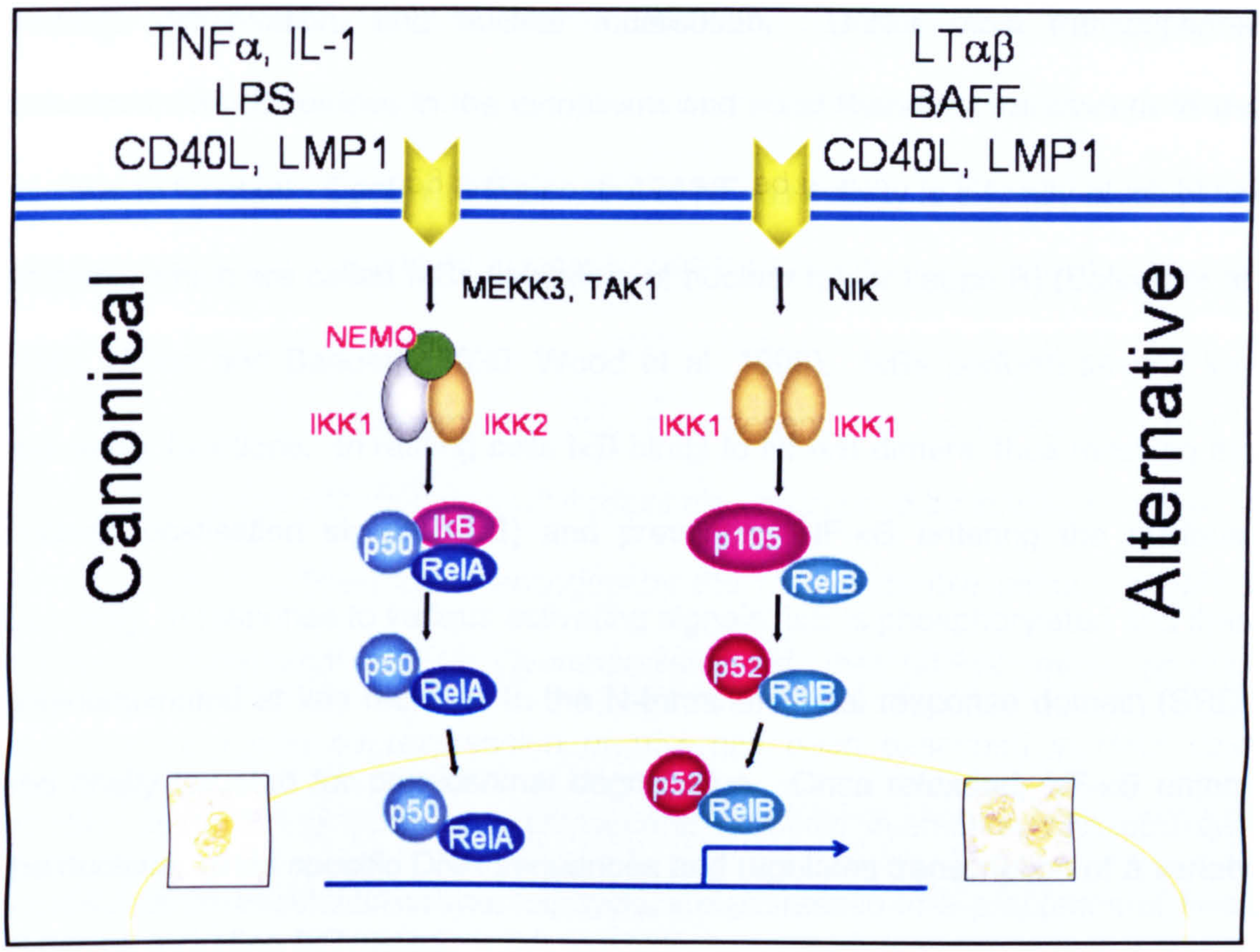

Figure 1.11 Activation of NF-kB by canonical and alternative pathways.

Aberrant NF-κB activity, leading to perturbation of cell cycle and apoptosis control mechanisms, is a feature of numerous human cancers including HL (Figure 1.11). The observation that HRS cells lack Ig expression suggests that they are derived from germinal centre B cells that should have been negatively selected (Kuppers and Rajewsky 1998), but were rescued from apoptosis by aberrant NF-κB activation or other mechanisms. NF-κB exists in virtually all cell types in the form of dimeric complexes consisting of different members of the Rel family of proteins (Ghosh et al. 1998). In mammals, there are five Rel proteins, p50, p52, p65, c-Rel, and Rel B, all of which share an amino-terminal 300 amino acid conserved region

known as the Rel Homology Region (RHR). This region is responsible for DNA binding, dimerisation, and nuclear localisation. Unlike most transcriptional activators, NF-κB resides in the cytoplasm and must therefore translocate to the nucleus to function. Central to the control of NF-κB activity is a family of inhibitory proteins, which are called IκBs (inhibitors of nuclear factor kappa B) (Ballard et al. 1990; Urban and Baeuerle 1990; Wood et al. 1998). IκBs perform several key regulatory functions. In resting cells IκB binds to NF-κB dimers, thus masking the nuclear localisation signal (NLS) and preventing NF-κB entering the nucleus. Secondly, in response to various activating signals, IκB is phosphorylated and then polyubiquinated at key residues in the N-terminal signal response domain (SRD) and finally targeted for proteosomal degradation. Once released, NF-κB enters the nucleus, binds specific DNA sequences and regulates transcription of a variety of genes, including *IkB* genes.

The IκB family consists of several members, IκBα, IκBβ, IκBε and Bcl-3. The carboxy-terminal regions of the precursors for p50 and p52, p105 and p100, respectively, can also function as IκBs. Depending on the cell type and on the stimulus, IκBs respond differently to NF-κB-inducing signals. In general, IκBα is rapidly degraded, whereas IκBβ and IκBε are degraded with slower kinetics. In addition, IκBs inhibit NF-κB with different efficiencies. For instance, IκBα is a stronger inhibitor of NF-κB than is IκBβ or IκBε (Hoffmann et al. 2002).

IκB proteins are not only responsible for cytoplasmic sequestration of NF-κB in resting cells, but they also associate with NF-κB in the nucleus, where they inhibit NF-κB DNA binding and promote transport of NF-κB to the cytoplasm, thus terminating transcription and restoring the cell to its original state (Simeonidis et al. 1999).

NF-κB is constitutively active in HRS cells (Bargou et al. 1996). Studies on transfected HL-derived cell lines suggest that the inactivation of NF-κB restores the sensitivity of HRS cells to apoptosis, pointing to the possibility that NF-κB plays an important role in obstruction of the apoptotic pathway (Wu et al. 1996). The mechanisms underlying this phenomenon could include constitutive activity of kinases upstream of IκBs, mutation or loss of *IkB*s, or modification of NF-κB rendering it insensitive to inhibition by IκBs. Amplification of NF-κBs has also been observed in many tumours, including HL (Bargou et al. 1997). Several reports now confirm that mutation of the *IkBa* gene and loss of protein is a culprit in a subset of HL cases (Cabannes et al. 1999; Emmerich et al. 1999; Jungnickel et al. 2000). Our group previously identified a large genomic deletion in a HRS cell-enriched population from a patient with relapsed HL. This deletion would result in mutant IκB that would be incapable of binding NF-κB and inhibiting NF-κB-dependent transcription. In HL-derived cell lines and patients that have wild-type *IkBa* alleles and express IκBα proteins, constitutive activity of IκB kinases (IKK), leading to rapid degradation of IκBα, may account for the presence of NF-κB in the nucleus. Although the cytokines secreted by the HL-derived cell lines can induce

nuclear NF-κB, the possibility of molecular defects in the components of the IKK cascade cannot be neglected (Krappmann et al. 1999). It has also been shown that the EBV-encoded protein latent membrane protein 1 (LMP1) can activate NF-κB by mimicking an activated CD40 molecule and thereby promoting IκBα turnover (Sylla et al. 1998).

Doerre and Corley (1999) have demonstrated that different B-cell lines use distinct strategies for the nuclear translocation of NF-κB, and that these differences may be associated with different isotypes of the B cell receptor. They observed stability of IκBα, IκBβ and IκBε in the B cell lines which contained constitutive nuclear NF-κB, including complexes containing RelA and c-Rel that are normally susceptible to cytoplasmic retention by IκBα. The presence of these complexes is in striking contrast to other cells, such as pre-Bs and T cells, that have stable IκB proteins but do not contain activated NF-κB. The authors therefore concluded that an alternative mechanism for the nuclear translocation of NF-κB which is independent of the degradation of IκBα, IκBβ and IκBε must exist (Doerre and Corley 1999).

1.11.2 Tumour Necrosis Factor Receptor and Tumour Necrosis Factor Ligand Superfamily Expression in HL

CD30, a member of the TNFR superfamily, was initially identified as a HL-associated antigen (Schwab et al. 1982). Subsequently, it was shown that CD30 expression is not specific for HRS cells, rather, it is a late activation marker for lymphoid cells, with normal CD30 expression being restricted to antigen-stimulated and memory T cells (Ellis et al. 1993). Nearly all HL-derived cell lines and HRS

cells in 85% to 90% of cases of cHL express CD30 (Drexler and Minowada 1992). An exception is the NLPHL subtype, in which the tumour cells do not express CD30 (Drexler 1992; Harris 1999).

The HRS cells also express several other members of the TNFR superfamily, including CD40, FAS, TNFR-1, TNFR-2, and 4-1BB. Additionally, primary HRS cells express members of the TNFR ligand superfamily, including TNF, lymphotoxin (LT) -α, CD27L, and CD30L (Gruss et al. 1996). The expression of multiple TNFR and TNF ligand superfamily members in HRS cells with their potential shared signal transduction pathways is further complicated by the fact that EBV LMP1 is a signaling homologue of the TNFR superfamily (Izumi et al. 1997).

1.11.3 Tumour Necrosis Factor Receptor Superfamily Signaling Pathways

There are multiple signal-transducing molecules that have been identified that interact with domains of TNFRs as well as with domains of other proteins that are directly involved in signal transduction. These so-called adaptor molecules, in general, have more than one family member, each of which functions in a slightly different manner and ultimately leads to transduction of different signals. The TNFR superfamily signaling pathways are shared with CD30, CD40, TNFR-1, TNFR-2 as well as EBV LMP1. The family of adaptor molecules called TNFR-associated factors (TRAFs) has at least six members, TRAF1 through to TRAF6 (Baker and Reddy 1996). The 230-bp TRAF domain mediates a number of specific protein-protein interactions (Takeuchi et al. 1996). TRAF1 has been

implicated as an important molecule in modulating NF-κB activation and it is overexpressed in the HRS cells and L&H cells of all HL cases studied (Durkop et al. 1999). In view of the findings that TRAF1 overexpression in transgenic mice inhibits antigen-induced apoptosis in CD8 positive T lymphocytes, it can be speculated that a deregulation of the TRAF1 gene may contribute to the blockage of apoptosis in HRS cells and L&H cells (Lee et al. 1996).

TRAF2 mediates both NF-κB activation and activation of the c-jun N-terminal kinase (JNK) pathway. TRAF2 is itself activated by TNFR1, TNFR2, CD40, CD30 and EBV LMP1 (Hsu et al. 1996). TRAF5 has been demonstrated to similarly activate NF-κB by members of the TNFR superfamily (Nakano et al. 1996). The mechanism of activation of NK-κB signaling is through the recruitment and activation of a series of protein kinases, which leads to the translocation of activated NF-κB into the nucleus of the cell. Although TRAF molecules have no intrinsic catalytic capability, their interaction with certain kinases appears to stimulate the activity of those molecules. The NF-κB-inducing kinase (NIK) is a mitogen-activated protein (MAP) kinase kinase kinase (MAP3K) (Malinin et al. 1997). A substrate of NIK is the IKK, which consists of two subunits IKKα and IKKβ. As mentioned previously, the IKK complex is essential for the phosphorylation and inactivation of IκBs (Regnier et al. 1997). This can then prevent HRS cells from undergoing stress-induced apoptosis.

In addition to NIK, TRAF2 has been shown to activate mitogen-activated protein kinase/ERK kinase kinase-1 (MEKK1). MEKK1 is a central kinase in the c-jun

activation pathway. There is an overlap of the two TRAF2-mediated pathways at MEKK1; MEKK1 has been shown to be able to phosphorylate IκB, leading to the activation of NF-κB (Lee et al. 1997). Thus, TRAF2 serves as an important branch point for the NF-κB and JNK pathways. Furthermore, the participation by MEKK1 in each of these signaling pathways provides the opportunity for these pathways to interact directly. The role of each of these pathways in mediating the downstream effects of TNFR family signaling is largely understood through experiments performed with dominant negative mutations of multiple members of the signaling molecules as well as knock-out transgenic mice. The results of these types of study suggest that the activation of NF-κB can protect the cell against TNF induced apoptosis. Also JNK activation is not directly linked to the induction of TNFR1-mediated apoptosis. The expression of a TRAF1-dominant negative in transgenic mice *in vivo* suggests that TRAF2 is required for the activation of JNK, but not NF-κB, through TNFR or CD40. These experiments also showed that TRAF2 has an anti-apoptotic affect that is independent of its ability to activate NF-κB (Lee et al. 1997; Yeh et al. 1997).

TRAF3 is unique among the TRAF family members in that this protein is capable of blocking TNFR family member-mediated activation of NF-κB. Furthermore, TRAF3 can block the ability of TRAF2 to activate this pathway. TRAF3 knock-out mice do not show significant defects in CD40 signaling. These mice, however, do show postnatal lethality and have defective T-cell-mediated immune responses (Xu et al. 1996). This suggests that TRAF3 is important in regulating certain cellular events during development. Although the effects of TRAF3 have not been

directly studied regarding JNK activation, the above suggests that TRAF3 may play a role in determining the ability of TRAF2 to activate the NF-κB or JNK pathway.

The ability of multiple members of the TNF receptor superfamily such as CD30, CD40, or TNFR1 to interact with various members of the TRAF family of signal transduction molecules and their coexpression in HRS cells may result in a complex cascade of signaling events in HL. The ability of the EBV LMP1 protein to interact with TRAF family members and activate these pathways also adds a level of complexity to the potential signaling processes in EBV-positive cases of HL. The notion that LMP1 may exert effects on HRS cell growth *in vivo* through TNF receptor signaling pathway is strengthened by the demonstration of TRAF-mediated LMP1 signaling *in vivo* in EBV-positive AIDS-related NHLs and post-transplant lymphoproliferative disease (Liebowitz 1998). The coexpression of multiple members of the TNF receptor superfamily, which all share components of the same signaling pathways, suggests that receptor cross-talk may be an important element in determining the phenotype of the HRS cells and the microenvironment of HL.

1.11.4 CD40 and CD40 ligand

CD40 is a 50-kd phosphoprotein expressed mainly on cells of B lineage, including most B-cell leukaemias and lymphomas (Law et al. 1990). CD40 acts as a receptor for a specific ligand (CD40L), which is a type 2 integral membrane glycoprotein that has homology to ligands for other receptors of the neuronal

growth factor (NGF)/TNF receptor superfamily (Smith et al. 1994). CD40L is expressed on activated CD4 helper T cells, including the cells that form rosettes around HRS cells. It is also expressed on some mast cells and basophils. Engagement of CD40 antigen by CD40L, or some CD40 monoclonal antibodies, results in the prevention of apoptosis of germinal centre B cells (Holder et al. 1993). CD40L is directly mitogenic for human B cells. Human B cell precursors require IL-3 as a co-stimulatory cytokine, whereas IL-4 enhances proliferation of mature B cells. It has been shown that LMP1 can mimic a constitutively active CD40 receptor, a signaling pathway leading to the activation of NF-κB in antigen-activated B cells (Gires et al. 1997).

CD40 is expressed at high levels on primary and cultured HRS cells. Therefore, engagement of CD40 might be expected to modulate the growth of HRS cells. Exposure of L428 and KM-H2 cells to different concentrations of soluble human CD40L resulted in a dose-dependent enhancement of their clonogenic growth and a striking increase in their colony size. These effects were enhanced by addition of IL-9. CD40L also enhanced expression of co-stimulatory and intercellular adhesion molecules ICAM-1/CD54 and B7-1/CD80 in HL-derived cell lines and induced release of the cytokines IL-8, IL-6, TNF, and LT-α (Gruss et al. 1994). Further studies of CD40L and agonistic anti-CD40 antibodies in animal models are needed to determine the *in vivo* consequences of engagement of CD40 on HRS cells.

1.12 Cytogenetic studies in non-Hodgkin lymphoma and Hodgkin lymphoma

Cytogenetic analysis has been extremely productive in the investigation of NHL, where the identification of non-random chromosomal abnormalities has led to the discovery of numerous oncogenes. In FCL, the investigation of tumour cells carrying the t(14:18) translocation led to the discovery of Bcl-2 (Tsujimoto et al. 1984). This translocation juxtaposes the IgH gene on chromosome 14 with the *bcl-2* gene on chromosome 18, resulting in dysregulation of Bcl-2. Investigation of this translocation has led to an improved understanding of the biology of both FCL and apoptosis, and detection of t(14;18) has proven useful both diagnostically and in clinical follow-up. Similarly, the identification of the t(2:5) translocation in ALCL allowed production of antibodies to the fusion protein NPM/ALK and the recognition of a discrete subgroup of patients with ALK-positive lymphoma or 'ALKoma' (Le Beau et al. 1989). In contrast to NHL, primary karyotypical analysis of HL has been frustrating, and some of the abnormalities observed may not be truly derived from the tumour cells. Reasons for the failure of these analyses include the paucity of the HRS cells or their variants in tissue samples, the lack of knowledge about their growth requirements *in vitro*, and the practical limitation of obtaining and analysing only a limited number of metaphases with routine classical banding cytogenetic techniques. Fluorescence immunophenotyping and interphase cytogenetics as a tool for investigation of neoplasms (FICTION) and other molecular techniques may be useful at the investigational level, but they are limited to analysis of specific abnormalities (Weber-Matthiesen et al. 1992). Prognostically significant chromosomal abnormalities and genes that may be

responsible for malignant transformation cannot be identified without global karyotyping.

The reported abnormal karyotypes in HL are extremely complex, and polyploidy is very common. Most abnormal karyotypes are characterized by multiple chromosomal gains or losses, translocations, inversions, and deletions. Material from 391 patients with HL was reviewed and included 294 analysable metaphases (Sarris et al. 1999). Overall, normal diploid karyotypes were seen in 142 patients (48%) with analysable metaphases (Koduru et al. 1989; Banks et al. 1991; Schlegelberger et al. 1994). This observation has fuelled the controversy about whether these normal karyotypes arise from the reactive mononuclear infiltrate, or whether HRS cells may have normal karyotypes in some patients. Abnormal metaphases, defined as those with either numerical or structural chromosomal abnormalities, were seen in 152 patients (52%) with analysable metaphases. However, in individual series the frequency of abnormal metaphases ranged from 13% to 92%. This extreme variability probably reflects methodological difficulties, including the analysis of samples that have variable cellularity or contain different numbers of malignant cells. In addition, differences in elapsed time from biopsy to placing the cells in culture, the exact culture conditions used to generate metaphases, and the exact banding techniques can all affect the number of abnormal metaphases observed.

Most of the abnormal karyotypes were hyperdiploid with several extra chromosomes. There was a broad distribution of chromosome numbers; the

highest reported chromosome number was 119, which corresponds to a hyperpentaploid cell. When gains or losses of individual chromosomes were analysed, the gains exceeded losses for most chromosomes. The exceptions were chromosomes 13, 15, 22, and Y, which are lost more often than they are gained (Falzetti et al. 1999; Pedersen et al. 1999). The reported structural abnormalities included translocations, inversions, deletions, or duplications. When the relative size of the p and q arms of each chromosome was taken into consideration, the data suggested that 2p, 3q, 6q, 7q, 9p, 13p, 14p, and 17q are altered much more than expected from their sizes, relative to the contralateral arm of the same chromosome (Sarris et al. 1999). This is particularly striking when chromosomes 9p and 13p are considered, because the p arms are much smaller than the q arms in these two chromosomes. These data are consistent with the presence of tumour suppressor genes that are inactivated, and/or tumour promoter genes that are activated by these complex chromosomal alterations. Numerous unidentifiable marker chromosomes resulting from complex rearrangements involving several chromosomes were commonly seen, and these may mask significant genomic abnormalities (Banks et al. 1991). These observations do not prove that expression of genes contained in these chromosomal loci has been altered. They may, however, form the basis for future research to investigate whether these genes are altered in HRS cells, and to identify candidate genes that may rescue HRS cells from apoptosis.

The t(14;18)(q32;q21) was characterized initially in FCL, where it was shown to result in the dysregulation of Bcl-2 production by juxtaposing the *IgH* locus on

14q32 with the *bcl-2* locus on 18q21. The bcl-2 locus encodes a mitochondrial protein whose overexpression protects lymphoid and non-lymphoid cells from apoptosis (Miyashita and Reed 1993). Extensive studies have documented the presence of t(14;18) in most FCL (Tsuijimoto and Croce 1986). Since HRS cells are derived from B cells with rearranged Ig genes, it was reasonable to probe for the presence of t(14;18) in HL. Several lines of evidence including classical cytogenetics, PCR including single HRS cell analysis, and fluorescence *in situ* hybridization (FISH) suggest that t(14;18)(q32;q21) is not a primary event in the molecular pathogenesis of HL (Sarris et al. 1999) (see section 1.12).

Since its initial description, ALCL (Stein et al. 1985) has been recognized as a distinct clinicopathological entity. ICC investigations reveal that both ALCL and HL express CD30 (Schwab et al. 1982). Among malignant lymphomas, only ALCL and HL express c-kit, the cellular receptor for stem cell growth factor (Pinto et al. 1994). The presence of CD30, c-kit, and sclerosis in both ALCL and HL has made the distinction between those disorders difficult.

By classical cytogenetics the t(2;5)(p23;q35) was frequently detected in ALCL (Le Beau et al. 1989), but neither 2p23 or 5q35 alterations have been reported in HL karyotypes with any frequency. The t(2;5) fuses sequences from the *NPM* gene, which is located on chromosome 5q35, to a novel gene, designated *ALK*, on chromosome 2p23. The *NPM* locus is highly conserved and codes for a nuclear phosphoprotein that is involved in late stages of ribosomal assembly (Chan et al. 1989). The *ALK* locus codes for a novel transmembrane protein kinase that has

sequence homology to the (missing) chain of the insulin receptor, the (missing) chain of the insulin-like growth factor-1 receptor, the leukocyte tyrosine kinase, and the Drosophilia homologue Sevenless. The fusion protein generated by the t(2;5)(p23;q35) consists of NPM amino-terminal sequences fused to ALK carboxyl-terminal cytoplasmic sequences, which include the consensus tyrosine kinase residues. Gene transduction experiments have demonstrated that this fusion protein is sufficient for malignant transformation (Kuefer et al. 1997).

In a meta-analysis the overall frequency of t(2;5) among 592 cases of HL was 4% which is much lower than the 37% frequency reported for 537 patients with ALCL (Sarris et al. 1999).

The generation of t(2;5) only in T-cell or null-cell ALCL but not B-cell lymphoma, the derivation of HRS cells from germinal centre B-cells, the negative results by classical cytogenetics, the predominantly negative results with molecular genetics, and the negative results with ICC methods suggest that t(2;5) is not a primary event in HL. There are many other translocations frequently found amongst other NHLs, such as t(8:14)(q24;q32) involving MYC-IgH, (3q27) involving Bcl-6, t(11:14) involving Bcl-1 or CCND1, t(9:14)(p13;q32) involving PAX5, t(14:15)(q32;q11-13) involving IgH-Bcl-8, Bcl-9, MUC1, t(11;18)(q21;q21) involving API2-MLT1, and t(1;14)(p22;q32) involving Bcl-10 but none of these has been studied in HL (Macintyre et al. 2000).

1.12.1 FICTION analysis in HL

FICTION is a technique that was developed to focus on the cytogenetic analysis of the malignant cells within tumours. This technique takes advantage of the frequent expression of various antigens, such as CD30, on HRS cells. Specific staining coupled with the size and number of nuclei allows the identification of HRS cells, since CD30 is rarely expressed by the reactive mononuclear cell infiltrate. The cytogenetic composition of these cells can then be determined with FISH using DNA probes specific for either a chromosomal centromere (numerical abnormalities) or for a specific DNA region (structural abnormalities). FICTION is limited to those cases where the HRS cells can be identified by CD30, and cannot be used to determine a global karyotype since it can only probe for aberrations in a specific chromosome, or for deletions or translocations for which there are available fluorescent probes. To date, results from FICTION studies have largely confirmed previous observations of hyperploidy in the HRS cells but specific recurrent chromosomal abnormalities were not identified until the advent of CGH (Weber-Matthiesen et al. 1995a; Weber-Matthiesen et al. 1995b).

1.12.2 Comparative genomic hybridisation

Comparative genomic hybridisation (CGH) is a molecular cytogenetic technique that screens for whole genomic imbalances in tumour samples. It identifies chromosomal gains and losses (e.g. duplications, amplifications or deletions) by using differentially labelled tumour DNA and normal DNA (Kallioniemi and al. 1992). CGH is based on quantitative two-colour FISH (Kallioniemi and al. 1993). It has become an invaluable technique for studying chromosomal aberrations that

occur in solid tumours and other malignancies (Zitzelsberger et al. 1997). Such studies have not only provided a basis for the identification of genes relevant for the pathogenesis of tumours, but have also contributed to recently developed tumour classifications (Heselmeyer and al. 1996; Joos et al. 1996; Kovacs et al. 1997; Nigro et al. 2001; Verdorfer et al. 2001; Tarkkanen and Knuutila 2002). There are numerous examples to date: Forozan et al. (2000) studied 38 established breast cancer cell lines by CGH to identify recurrent genetic alterations and determine the extent to which these cell lines resembled uncultured tumours. A comparison of DNA copy number changes found in the cell lines with those reported in 17 published studies (698 tumours) of uncultured tumours revealed a substantial degree of overlap. CGH copy number profiles may facilitate identification of important new genes located at the hotspots of such chromosomal alterations. This was illustrated by analysing expression levels of 1236 genes using cDNA microarrays in four of the cell lines. Several highly overexpressed genes (such as *RCH1* at 17q23, *TOPO II* at 17q21-q22, as well as *CAS* and *MYBL2* at 20q13) were involved in these recurrent DNA amplifications. Zitzelsberger et al. (2001) studied 16 prostate carcinomas, 12 prostatic intraepithelial neoplasias (PIN; 4 low-grade and 8 high-grade) adjacent to the invasive tumour areas, and 5 regional lymph node metastases. The pooled CGH data from the prostatic carcinomas revealed a novel region of chromosomal loss on 4q, a region which is also frequently affected in other tumour entities such as oesophageal adenocarcinomas. This region may therefore harbour a novel tumour suppressor gene. Gains on chromosomes 9q

and 16 and loss on chromosome 13q were observed as common aberrations in metastases and primary tumours. These CGH results indicate an accumulation of chromosomal imbalances during the PIN to invasive carcinoma to metastasis sequence and an early origin of tumour-specific aberrations in PIN areas. Heselmeyer et al. (1996) studied tissue from the cervical epithelium at various stages of dysplasia. Their results showed that gain of chromosome 3q defines the transition from severe dysplasia to invasive carcinoma.

1.12.2.1 Advantages of CGH

A major advantage of the CGH technique is that only DNA from tumour samples is needed for analysis; this avoids the often-difficult preparation of tumour metaphase chromosomes, which can have poor morphology and resolution. Instead, karyotypically normal metaphase chromosomes are used to detect tumour-associated chromosomal gains and losses. Another advantage of CGH is that formalin-fixed tissue sections can be used; thus, comparisons can be made between a phenotype and genotype, and genetic changes can be correlated with the clinical course of a disease.

1.12.2.2 Limitations of CGH

The CGH technique will not be able to detect balanced chromosomal rearrangements, such as reciprocal translocations or inversions. CGH is further limited by the resolution of altered chromosomal regions. Resolutions for amplifications and deletions of 5-10 Mb might reasonably be expected when analysing tumour material in practical experiments due to varying condensation of

metaphase chromosomes, intratumour heterogeneity and contamination with normal cells (du Manoir et al. 1995). The reliability and sensitivity of CGH analysis is dependent on the homogeneity of the isolated tumour DNA sample. If the tumour sample contains >50% non-tumourous cells, a significant number of imbalances may not be detected. Due to the high variability of CGH technique and limitations of methodology, it is important to validate chromosomal changes detected by CGH by other methods, such as FISH, loss of heterozygosity analysis or conventional cytogenetics.

1.12.2.3 Outline of CGH

For CGH, whole-genomic DNA is isolated from a tumour by standard extraction protocols. For single-cell work, whole genome amplification techniques such as degenerate oligonucleotide primed PCR (DOP-PCR) can be used to provide sufficient quantities of DNA. Control or reference DNA is isolated from an individual who has either a normal 46, XX karyotype or a normal 46, XY karyotype. The DNA that has been extracted from the two genomes is differentially labelled (for example, using biotin conjugated to dUTP for the tumour genome and digoxigenin conjugated to dUTP for the normal genome). The tumour and normal DNA samples are combined, and an excess of unlabelled human CoT-1 DNA is added into the hybridisation mixture, to suppress the repetitive sequences that are present in both genomes. The CoT-1 DNA is essential because hybridisation of the repetitive DNA would impair the evaluation of the unique sequences that are either overrepresented or underrepresented in the tumour genome. This probe mixture is hybridised to normal human reference metaphase chromosomes. With

indirect labelling, avidin coupled with fluorescein isothiocyanate (FITC), which fluoresces green, is used for the detection of bound biotin-labelled tumour DNA, whereas antidigoxin coupled to rhodamine, which fluoresces red, is used for the digoxigenin-labelled control DNA. It is also possible to use direct labelling with SpectrumGreen and SpectrumRed, respectively. The relative colour intensities of the two fluorochromes reflect DNA copy-number alterations in the tumour genome (McNeil and Ried 2000).

To determine copy-number imbalances within a tumour, images must be acquired from several metaphase spreads using a charged-coupled device (CCD) camera. Variations in the fluorochrome intensity along the chromosomes in a metaphase spread reflect copy-number changes (i.e. gains and losses of DNA along a chromosome) in the tumour sample. For example, using the fluorochromes mentioned above, a loss of DNA within the tumour genome shifts the colour of that region to red. A gain of a chromosomal region is shown by an increased intensity of green fluorescence in the reference metaphase preparation. If chromosomes or subchromosomal regions are balanced with respect to DNA content in both tumour and control samples, the intensity of the red and green fluorescence is similar. Digital image analyses must be used to quantify fluorochrome intensity, especially in cases where changes involve low copy numbers or where many gains and losses have occurred. Specialised software selects metaphases that have adequate signal intensity, aligns them along the chromosomal axis, and correctly identifies and orientates each chromosome. For each chromosome, a ratio value of fluorochrome intensity is generated from a minimum of 5 to 10 metaphase

spreads. Finally, an average ratio profile for each chromosome is produced, indicating the calculated gains and losses (Piper et al. 1995).

1.12.2.4 CGH and HL

Two research groups have carried out CGH analysis on single or small numbers of HRS cells, using slightly different approaches. Ohshima et al. (1999) performed CGH on 9 cases of HL with HRS cells isolated by flow cytometry based on cell size and CD30 staining. They identified the most common genetic aberrations as gains on 1p13 and 7q35/36 and loss of 16q11/21. Subsequent analysis of loss of heterozygosity (LOH) on 16q revealed a discrete region, 16q21-23, that was frequently deleted. It was suggested that loss of E-cadherin might be involved in the formation of HRS cells (Ohshima et al. 2001). Joos et al. (2000) performed CGH on 12 cases of HL with the HRS cells isolated by micromanipulation using micropipettes made from glass capillaries. The selection was based on morphology alone on frozen sections. A number of recurrent chromosomal imbalances were identified with the suggestion of amplification of MDM2 and JAK. Both groups identified recurrent gains and losses and areas of high amplification but there was little agreement between the two studies. Thus, the main aim of this project was to carry out further CGH studies in HL to elucidate the genetic changes in the HRS cells of HL.

Chapter 2

MATERIALS AND METHODS

2.1 Chemicals

All chemical reagents used were of Analar or molecular grade and were purchased from Sigma Aldrich (Poole, UK) or BDH (Poole, UK) except where specifically stated. Deionised water obtained from a reverse osmosis system (Millipore, Livingston, UK) was used for making up buffers and solutions. Deionised, filtered water (MilliQ water filtration system, Millipore) was used in enzyme-catalysed reactions and also for the dissolution of DNA and proteins.

2.2 Tissue samples

Freshly removed lymph node biopsies were delivered to the laboratory on the same day or next day by courier service. The specimen was placed in travelling medium (see Appendix) in a plastic container and delivered in a specially designed box, approved by the Post Office. A data sheet with relevant clinical information, filled in by the submitting pathologist, was also included.

2.2.1 Processing of tumour biopsies

In order to preserve viability of specimens, the specimens were processed as quickly as possible. The entire process was carried out in a Containment level II facility within a Class II microbiological safety cabinet and according to the local Code of Practice. Clinical information was entered into the LRF computer database and a unique patient number assigned. Lymph node processing was carried out by several members of the group, including myself.

2.2.2 Preparation of viable cell suspensions

The biopsy sample was cut in half and imprints were made on Superfrosted microscope slides (BDH). Two slides were air-dried and two were fixed in acetone for 10 minutes. The tissue was then cut into smaller pieces with a pair of scissors, placed into a Medicon cassette (Dako, Ely, UK) and mechanically disrupted using a MediMachine (Dako). The cells were resuspended in a small amount of travel medium (see Appendix) and removed from the cassette using a 5 ml syringe. The Medicon was washed twice to ensure complete removal of all cells.

2.2.3 Cell counting

Twenty microlitres of cell suspension was mixed with 20 μl of Trypan blue dye (Sigma) in a microfuge tube (Scotlab Ltd., Strathclyde, UK). Approximately 20 μl of the mixture was placed on a haemocytometer (VWR International Ltd., Poole, UK), beneath a coverslip. The total number of viable cells in 16 large grid squares was counted. This number was multiplied by the dilution factor, giving the concentration x 10^4 per ml.

2.2.4 Enrichment of mononuclear cells from lymph nodes

The cell suspension was slowly layered onto Lymphoprep (Nycomed, Oxford, UK) in a 15 ml Falcon tube (see Appendix) for gradient density centrifugation. The suspension was centrifuged in a bench top centrifuge (Allegra GR, Beckman Coulter, High Wycombe, UK) at 1000 x g for 30 minutes with no brake applied. The middle layer, containing the mononuclear cell fraction, was then removed carefully with a pipette and resuspended in 10 ml Hanks buffered salt solution

(HBSS) (Invitrogen, Paisley, UK) + 2% fetal bovine serum (FBS) (Invitrogen) or MiniMacs buffer (see Appendix) + 2% FBS. A cell count was performed and then the cells were washed once more followed by centrifugation at 1000 x g for 5 minutes with braking.

2.2.5 Storage of viable mononuclear cell suspensions

The cell pellet was resuspended in 1 ml of 92% FBS + 8% DMSO (see Appendix) (usually 10^7 cells/ml) and aliquoted into 1.5 ml Nunc tubes (Nalge, Hereford, UK). The tubes were labelled and stored overnight at -80°C in a Nalgene Cryo1°C freezing container (Nalge) and then placed in long term storage in liquid nitrogen. Sample details and storage location were recorded in the LRF database.

2.3 Cell lines

Cell lines, including HL-derived cell lines, were used for optimisation of experimental protocols and as controls. The cell lines used during this research project were L428, L591, KM-H2, Raji, Daudi and IM9 (see Appendix). They all have very similar growth requirements which allowed them to be cultured in the same way.

2.3.1 Maintenance of tumour-derived cell lines

Aliquots of viably-frozen cell lines were removed from liquid nitrogen storage and thawed at 37°C. As soon as the cells had thawed they were gently diluted into 10 ml HBSS + 2% FBS. The cells were pelleted by spinning at 1000 x g for 5 minutes in a bench top centrifuge (Allegra GR, Beckman Coulter) and then resuspended in

10 ml PBS buffer to remove DMSO. A cell count was performed and, following a further washing step, the cells were resuspended in an appropriate volume of cell culture medium (see Appendix) in aerated culture flasks. The cell cultures were incubated at 37°C in 5% CO_2, and were passaged twice per week. See section 2.5 for extraction of DNA from cells.

Handling of cell lines was carried out in class II microbiological safety cabinets. It was routine practice to handle EBV-negative cell lines prior to EBV-positive ones.

2.4 ICC and *in situ* hybridisation

ICC was used to positively identify HRS cells on plain or polyethylene naphthalate (PEN) foil (see section 2.7.1) cytospins of cell suspensions. Three different ICC techniques were tested in this research project. These included the Dako APAAP, Dako ABComplex and Dako CSA techniques. The Dako ABComplex was our favoured technique because of its relative ease of use, low background and high specificity. The primary antibodies that were used were HRS4 (Immunotech, Beckman) and Ber-H2 (Dako), which are both mouse monoclonal antibodies reactive with CD30.

2.4.1 ABComplex method for detection of CD30

Cytospins or imprints were stored at -80°C and had to be equilibrated to room temperature (RT) prior to use. All subsequent steps were performed at RT and slides were washed twice in 1x TBST (see Appendix) for 3-5 minutes between each step. An Immunopen (Dako) was used to circle the cytospin spots to minimise the amount of reagent used and avoid drying. The volume required to

cover the cytospin spots was 50 µl. Twenty percent rabbit serum (Vector Laboratories, Peterborough, UK) was applied to each cell spot to block non-specific staining and the slides were incubated for 20 minutes. CD30 primary antibody diluted to 1:100 was then added and the slides were incubated for 30 minutes. This was followed by a similar incubation period with a 1:300 dilution of a biotin-conjugated rabbit anti-mouse secondary antibody. An aliquot of ABComplex was added to each cell spot and the slides were incubated for a further 30 minutes. Freshly made FastRed substrate (Dako) was added to each spot and incubated for 10 to 15 minutes. The slides were washed twice in distilled water and then air-dried overnight in an aerated box. For preparation of plain glass slides, D.P.X. mountant (see Appendix) was used.

2.4.2 Deparaffinisation and antigen retrieval

Sections of paraffin-embedded tissues were cut at 3 µm thickness per section and mounted onto 3-aminopropultriethoxysilane (APES) (Sigma) treated slides. Sections were dewaxed in Citroclear (National Diagnostics, Atlanta, USA) and rehydrated in graded ethanols. Antigen retrieval was achieved by pressure-cooking slides in EDTA buffer (pH 8.0) for 160 seconds.

2.4.3 ABComplex method for detection of LMP1

EBV status of HL biopsies was determined by using LMP1 ICC or EBER *in situ* hybridisation on sections of paraffin-embedded material. For LMP1 detection, paraffin sections that had been through antigen retrieval and deparaffinisation were immersed in 0.2% glycine solution for 2 minutes followed by 70% and 90%

ethanol for 2 minutes each. This procedure utilised a Vectastain ABComplex kit (Vector Laboratories), which is similar to the Dako ABComplex kit. The main difference between the two methodologies is that horseradish peroxidase, rather than alkaline phosphatase, is the enzyme used in the Vectastain kit. Slides were therefore incubated in 1.5% hydrogen peroxide/methanol solution (see Appendix) for 10 minutes prior to the application of the blocking serum, in this case 20% horse serum. A 1:50 dilution of the CS1-4 cocktail of monoclonal antibodies (Dako), reactive with LMP1, was added to the section and the slides were incubated for 1 hour. The slides were washed and 50 μl of biotinylated anti-mouse secondary antibody was added and the slides incubated for 30 minutes. The ABComplex was then added followed by a 30-minute incubation. The cells were then incubated in 3,3'-diaminobenzidine (DAB) (Sigma), the chromogenic substrate, for 10 to 15 minutes. Finally, the slides were washed twice in distilled water prior to being counterstained with haematoxylin using a standard procedure. Slides were mounted in D.P.X. (see Appendix) and left to dry on a heat block at 70°C for 30 minutes.

2.4.4 EBER *in situ* hybridisation

Sections of paraffin-embedded material were immersed in 0.2% glycine solution for 2 minutes followed by 70% and 90% ethanol for 2 minutes each. The slides were air-dried and 15 μl of FITC conjugated EBV EBER probe (Novacastra, Newcastle, UK) and hybridisation buffer (Novacastra, Newcastle, UK) were added to each section. The sections were covered with a small coverslip and incubated in a dark, humid chamber at 37°C overnight. The coverslips were removed and

the slides were washed three times in TBS (see Appendix) for 3 minutes each. Fifty microlitres of 20% rabbit serum were added to each section and the slides were incubated for 10 minutes. The excess solution was blotted off and 50 µl of a 1:50 dilution of anti-FITC antibody conjugated to AP (Novacastra, Newcastle, UK) were added to each section and the slides were incubated for 30 minutes. After washing twice with TBS, the slides were immersed in substrate buffer (Novacastra, Newcastle, UK) for 3 minutes. Finally, 50 µl of NBT/Levamisole substrate solution (Novacastra, Newcastle, UK) were added and the slides were incubated for at least 40 minutes. Slides were washed with distilled water and mounted with aqueous mountant (Dako). The scoring was performed according to accepted criteria (Gulley et al. 2002).

2.5 Purification of high molecular weight DNA from eukaryotic cells

Cell pellets of cell lines or tumour samples were resuspended in 5 ml, or an appropriate volume, of TNE (see Appendix). Sodium dodecyl sulphate (SDS) and proteinase K (see Appendix) were added to final concentrations of 0.5% and 50 µg/ml respectively and the suspension was incubated at 56°C for 60 minutes. Two volumes of phenol (see Appendix) were added to the lysate and the phases mixed slowly for 10 minutes. Following centrifugation at 3000 g without braking for 10 minutes at RT, the aqueous phase was removed to a sterile tube using a wide-tipped polypropylene pastette (see Appendix). An equal volume of phenol/ chloroform /isoamylalcohol (PCI9) (see Appendix) was added, the phases mixed thoroughly and then separated as before. This step was repeated with chloroform containing 4% isoamylalcohol. A wide-tipped pipette was used to transfer the

aqueous phase into at least two volumes of ice cold 100% ethanol. High molecular weight DNA was spooled onto a sealed Pasteur pipette and transferred to a microfuge tube (Scotlab Ltd.) to be washed with 70% ethanol. The pellet was allowed to dry for 1-2 hours. The DNA pellet was resuspended in TE buffer (see Appendix) and incubated at 37°C overnight or until the DNA had completely dissolved.

The concentration and purity of the DNA solution were determined by measuring the optical density (OD) of the solution at wavelengths of 260 nm and 280 nm using a GeneQuant 2 spectrophotometer (Amersham, Buckinghamshire, UK). The concentration of the DNA solution was calculated on the basis that an OD_{260nm} of 1.0 corresponds to 50 μg/ml of double stranded DNA. The purity of the solution was determined by calculating the $OD_{260/280nm}$ ratio. A value of 1.5 to 1.8 was considered indicative of acceptable quality, free from contaminating proteins.

2.5.1 Ethanol precipitation of DNA

Ethanol precipitation was used to clean up and concentrate nucleic acids. One tenth volume of 3 M sodium acetate and 2 volumes of ice-cold 100% ethanol were added to the DNA solution and mixed well. The mixture was chilled to -20°C overnight or -70°C for 30 minutes, to allow the DNA precipitate to form. This was followed by centrifugation at maximum speed (12000 g) in a microfuge (Scotlab Ltd., Strathclyde, UK) for 30 minutes at 4°C. The supernatant was discarded and 250 μl of 70% ethanol was added, the mixture was vortexed briefly and centrifuged for 30 minutes at 4°C. The supernatant was discarded and the microfuge tube

was left in an inverted position on a layer of absorbent paper to allow as much of the supernatant as possible to drain away. Traces of supernatant were removed using a sterile cotton bud or by a brief spin in a SpeedVac (ThermoSavant, Cheshire, UK). Over drying of the pellet was avoided as this resulted in difficulty in re-dissolving the pellet. Finally, the pellet was resuspended in the desired volume of TE buffer or water.

2.6 Introduction to polymerase chain reaction

PCR is now firmly established as an important technique in many aspects of biomolecular research. The technique allows unlimited amplification of DNA fragments using primers complementary to the DNA sequence. All that is required is a small amount of DNA template, primers, thermostable polymerase, magnesium, nucleotides and repeated cycles of denaturation, annealing and extension. Several different types of PCR were used in this project. Real-time quantitative PCR (RQ-PCR) and conventional PCR are covered in this section. DOP-PCR protocols are discussed in section 2.8.

2.6.1 Real-time quantitative polymerase chain reaction

Real-time quantitative PCR (RQ-PCR) is a method for the reliable detection and measurement of products generated during each cycle of the PCR. In the exponential phase of the reaction, the rate of amplification is directly proportional to the amount of template prior to the start of the PCR. The most commonly employed methodology uses TaqMan hydrolysis probes and the ABI Prism 7700 Sequence Detection System (Applied Biosystems, Warrington, UK). The addition

of a hydrolysis probe to the reaction makes the RQ-PCR assay highly specific. An oligonucleotide probe is constructed with a fluorescent reporter dye bound to the 5' end and a quencher on the 3' end. While the probe is intact, the proximity of the quencher greatly reduces the fluorescence emitted by the reporter dye by fluorescence resonance energy transfer (FRET) through space. During PCR amplification, the 5' nuclease activity of the polymerase cleaves the 5' end of the target-specific fluorescent probe. This cleavage of the probe separates the reporter dye from the quencher, increasing the reporter dye signal. When excited by a light source, i.e. laser, the cleaved probe emits a signal at a wavelength due to FRET, which transforms the energy into light. The system was used to measure DNA copy number of cellular and viral genes. Data are expressed as CT values; the CT corresponds to the cycle number at which the amplification plot for a given sample crossed the threshold, which was set at the point where the fluorescent signal equaled 10 times the standard deviation of background fluorescence.

The TaqMan primer and probe sets used in this project were designed in-house. The software programme Primer Express (Applied Biosystems) was used to design the optimal combination of primers and TaqMan probes for each assay. The programme ensures the primers and probe have appropriate melting temperature, low GC content in the most 3' 5 base pairs (bp) and prevents selection of primers that will dimerize or form hairpins. Smaller amplicons (<80 bp) are preferable; these small amplicons enable the extension time of the reaction to be very short (around 15 seconds), and also enable the primers and probe to compete more effectively with the complementary target sequence. The optimal

concentration of primers was determined for each assay. Primers and probes used in this project are shown in Table 2.2 (see Section 2.8.1).

RQ-PCRs contained 5 μl of DNA template, corresponding to 6.5 to 360 pg DNA. Reactions were performed using TaqMan Universal PCR Master Mix (Applied Biosystems) in a final volume of 25 μl with primers and probe at an optimised concentration. Following initial incubations at 50°C for 2 minutes and 95°C for 10 minutes, 40 cycles of thermal cycling at 95°C for 15 minutes and 60°C for 60 seconds were performed.

2.6.2 Conventional PCR using small cell numbers

The *IkBa* gene was successfully amplified from small numbers of cells using a two-step procedure and a multiplex PCR kit (Qiagen, West Sussex, UK). Multiplex PCR was advantageous because it allows multiple assays to be performed on small samples, using identical conditions. A table of the primer combinations used is detailed below (Table 2.1). A list of the full sequence of individual primers is provided in the Appendix.

Exon	First Round		Second Round		Product size
	Forward	Reverse	Forward	Reverse	bp
1	699	720	746	719	290
2	593	702	593	745	176
3	721	694	684	694	446
4	722	696	723	696	236
5	724	726	725	726	417
6	703	708	703	627	450

Table 2.1 Identifier for *IkB*a exon 1-6 primer sequence sets (see Appendix for corresponding sequences).

The *IkBa* gene is made up of 6 exons. The first round was a multiplex PCR reaction and each exon was amplified individually in the second round. In these experiments, 10 to 20 HRS cells were lysed in 10 μl of TaqMan lysis buffer (see Appendix) for 1 hour at 50°C. This was added to 25 μl of Qiagen Multiplex MasterMix, which contained an optimised amount of HotStarTaq polymerase, $MgCl_2$, and dNTPs. Aliquots of 10x primer mix, containing 2 μM of each primer, were made by combining 6 pairs of first round primers. The reaction contained 2.5% DMSO and the total reaction volume was 50 μl. Thermal cycling was performed on a GeneAmp PCR System 9600 (Applied Biosystems) using the following cycling conditions for the first round of PCR: 95°C for 15 minutes followed by 35 cycles of 95°C for 30 seconds, 55°C for 90 seconds, 72°C for 90 seconds and a final extension at 72°C for 10 minutes. In the second round, 1 μl of first round product was added to 25 μl of Qiagen Multiplex MasterMix with 0.2 μM of second round primers in a reaction volume of 50 μl. For exon 1 only, the reaction contained 2.5% DMSO. Thermal cycling was performed in the same thermocycler using the following conditions: 95°C for 15 minutes followed by 35 cycles of 95°C for 30 seconds, 65°C for 30 seconds, 72°C for 90 seconds and a final extension at 72°C for 10 minutes. The products were visualised under ultraviolet (UV) following electrophoresis on 8% polyacrylamide gels (see section 2.6.3.2) and staining with ethidium bromide. Amplification products of the appropriate size were subjected to direct sequencing (see section 2.6.3.3).

2.6.3 Analysis of PCR products

2.6.3.1 Agarose gel electrophoresis

Agarose (see Appendix) was made up to 1% weight/volume or appropriate in 1xTBE or TBA buffer (see Appendix) and dissolved by heating in a microwave. The solution was cooled to 55°C and 2 μl of 0.5 μg/ml ethidium bromide added before pouring onto a horizontal Perspex bed (see Appendix) with a well-forming comb in position. Following solidification, the gel was submerged in an electrophoresis tank, covered with 1xTBE buffer and the well-forming comb removed.

Prior to electrophoresis, DNA samples were mixed with a one-tenth volume of loading buffer (see Appendix). Samples were loaded into wells and a constant potential difference of 4 - 8 V/cm applied across the gel for 30 minutes to 4 hours, depending on the application. The DNA was visualised on a UV transilluminator at 300 nm and photographed using a Polaroid MP4 Land camera with Polaroid Type 57 high speed film or captured digitally using UVIsave (Thistle Scientific, UK).

The size of DNA fragments were estimated by comparison with the migration distances of DNA fragments of known sizes. For this purpose, 0.5 – 1 μg of *Hind*III digested bacteriophage lambda DNA (Sigma Aldrich Ltd.) or *Hae*III-digested PhiX174 RF DNA (Sigma Aldrich Ltd.) were electrophoresed alongside each batch of DNA samples.

2.6.3.2 Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis (PAGE) was used for the separation of DNA fragments less than 1 kilobase (kb) in length. Polyacrylamide solutions were made up to a final concentration of 8% (Liquideacrylamide 30% and Bisacrylamide 2%) in 1xTBE and polymerised by the addition of 0.06% (w/v) ammonium persulphate (APS) and 0.03% TEMED (N, N, N, N – tetramethylethyldiamine). The gel (10 cm x 8 cm x 2 mm) was poured between glass plates (see Appendix) and allowed to polymerise for 30 minutes with the well forming comb in place. After polymerisation was completed, the well-forming comb was removed and the wells thoroughly flushed with 1xTBE. DNA samples in loading buffer were loaded onto the gel and electrophoresed at 12 V/cm for 60 minutes using 1xTBE as the running buffer. DNA fragments were visualised using UV light following staining of the gel in 0.5 μg/ml ethidium bromide in 1xTBE for 30 seconds.

DNA fragment sizes were estimated by comparison with the migration distances of DNA fragments of known size. For this purpose, 0.5 – 1 μg of *Hae*III-digested PhiX174 RF DNA were electrophoresed alongside each batch of DNA samples.

2.6.3.3 Sequence analysis

Following purification using the Quickstep system (Edge BioSystems, MD, USA), PCR products were sequenced directly without prior cloning. Sequence analysis was performed using the BigDye Terminators v3.1 Cycle Sequencing Kit and an ABI Prism 3100 Genetic Analyzer (Applied Biosystems). This part of the project was carried out by Ms. Annette Lake. Single nucleotide polymorphism (SNP)

assays to look for loss of heterozygosity were also performed by Ms. Annette Lake.

2.7 Laser microdissection of single cells

Advances in our knowledge of the biology of HL in recent years have been helped enormously by the ability to isolate HRS cells from surrounding tissue. Modern techniques, such as fluorescence activated cell sorting (FACS) and affinity-labelled magnetic bead separations (Miltenyi Biotec, Surrey, UK), allow separation of subpopulations from heterogeneous pools of single cells in suspension. Unfortunately, they do not allow morphological assessment which is crucial to the selection of HRS cells since there is no absolutely specific immunological marker for these cells. Microdissection techniques involving micromanipulation and suction of a cell through micron-sized glass pipettes, dissection using a piezo-activated metal knife followed by suction through a glass pipette, and dissection using lasers have therefore been developed for HRS cell isolation. The pros and cons of using tissue sections, cell suspensions and cytospins for HRS cell isolation are discussed in the following section.

Prior to these experiments, both the FACS and the Mini-MACS techniques were used at the LRF Virus Centre to enrich HRS cells. Microdissection using two hydraulic micromanipulators was used to obtain single HRS cells from single cell suspensions. The major disadvantage of all three techniques was the necessity for a large amount of viable starting material, freshly prepared on the day. FACS and Mini-MACS allow enrichment of a large number of CD30 or CD15 positive

cells but it is difficult to ensure high purity as the antibodies used react with a variety of other non-tumour cells. Single cell micro-manipulation is an inefficient technique in comparison since it takes a long time to obtain a small number of cells and is highly skill dependent. Although this technique allows direct visual assessment of morphology, identification of HRS cells in cell suspensions is not straightforward. The development of laser microdissection (LMD) instruments therefore offered a better and more user-friendly technique. Because of the high energy concentrated into a small area, the easy control of the beam position, and the lack of direct contact with the material to be dissected, lasers provide the ideal tool for easy-to-use, large-scale microdissection. The potential of LMD was also linked to the development of highly sensitive and specific molecular techniques, such as single cell PCR and whole genome amplification (WGA), which allow analysis of a relatively small number of cells.

Three commercially available systems were designed specifically for LMD: PixCell by Arcturus (California, USA), PALM by P.A.L.M. Mikrolaser Technologie (Bernried, Germany), and the Leica AS LMD by Leica (Milton Keynes, UK). Following demonstrations and discussions with other scientists, the Leica AS LMD was selected for use in the LRF Virus Centre as it is a third generation laser microdissection system and appeared the most user-friendly of the three systems. It uses a UV laser, similar to the PALM system, and therefore avoids problems of heating associated with the infrared laser in the PixCell system. The laser beam is moved with a software-controlled mirror system to select cells to be ablated or to isolate the area to be dissected. The dissected material is allowed to fall by gravity

into the cap of a PCR tube and may thereafter be used for isolation of proteins or nucleic acid. The PALM system differs from the Leica system in having a static laser beam and having to catapult the dissected cells upwards into a collecting cap.

2.7.1 PENfoil slides for LMD

Regular microscope slides may be used in all systems, albeit with suboptimal results. For the PALM and the Leica AS LMD, it is recommended that a thin PEN (polyethylene naphthalate) foil (PALM) is mounted between the slide and the tissue specimen. The PENfoil is cut by the ablation laser around the area targeted for dissection and then catapulted in the PALM or let drop in the Leica system, thus preserving the integrity of the attached cells. Dissected pieces of tissue may be visualised after capture in the collecting cap only if the underlying membrane keeps the structure intact. If cells are catapulted directly from a slide, the material is pulverised and it is impossible to assess the efficiency of capture by visualisation. Lack of humidity may contribute to increased electrostatic forces that compete with gravity and affect collection in the Leica AS LMD system.

2.7.2 Optimised PENfoil slide preparation

All aspects of LMD were optimised as part of this project. A series of experiments was performed to determine: the optimal type of glass slide; the optimal sample fixation and staining; the optimal method for attaching PENfoil to slides (type of adhesive agent, number of edges to be sealed); and the optimal procedure to avoid static.

PENfoil membranes were cut to size (50 x 22 mm) and mounted on plain glass slides (BDH). Superfrost charged slides were found to be unsuitable as it was subsequently difficult to detach the PENfoil membrane from the slides. To ensure wrinkle-free application, the glass slide was first dipped in 70% alcohol after which the pre-cut membrane and backing paper were applied to the wet glass. The paper support was then removed and the membrane attached using Fixogum rubber cement (Marabu, Tamm, Germany) along two opposing edges. The slides were then left to dry for 2-3 days in a clean slide box. Cells from the Raji cell line were used to determine the optimal slide preparation method prior to using cHL samples.

2.7.3 Optimised cytospin preparation for LMD

Cytospins of cHL were made either from freshly processed lymph node cell suspensions, cell line suspensions or from viable cells stored in liquid nitrogen. Cytospins were preferred over frozen or paraffin-embedded tissue sections. The advantage is the ability to obtain high quality DNA from the whole cell, selected on the basis of both morphology and immunophenotype, without nuclear truncation.

Usually two vials of 10^7 cells were removed from liquid nitrogen, the cells were thawed (as described in Section 2.3.1) and a viable cell count performed. PENfoil coated slides and double chamber cytospin funnels were held together with a cytospin clip and loaded onto the cytospin holder. One hundred microlitres of cell suspension (2.5×10^5 cells) were loaded into each chamber. The cells were spun at 450 rpm for 10 minutes in a cytocentrifuge (Cytospin 2, ThermoShandon,

Cheshire, UK). The slides were removed quickly from the cytospin assemblies and air dried for 10 minutes. The cells were then fixed in ice-cold acetone for 10 minutes and air dried for a further 10 minutes. An Immunopen (Dako) was used to mark a circle around the cell spots on the slides. Slides were wrapped in aluminium foil and stored at -80°C until use.

If the diagnosis of cHL was suspected on receipt of a fresh sample, PENfoil slides were prepared from the fresh specimen following the above procedure.

2.7.4 Optimised conditions for LMD

Laser microdissection was carried out in a dedicated room where UV light was used to sterilise the area before and after each session. This also served to reduce static accumulation (see below). A recurring problem with the LMD setup was the effect of electrostatic build up from surrounding equipment and apparatus. The manufacturer recommended treating all plasticware, slides and equipment with UV light to reduce static. Treatment of PENfoil coated slides prior to cytospin preparation, ICC and storage was not efficacious. Treatment of our collection tubes with UV for at least 1 hour prior to LMD did help to reduce static.

Calibration of the Leica AS LMD system as recommended by the manufacturer was performed prior to each session. Cytospins of Raji cells were stained with the DiffQuik stain kit (ThermoShandon, Cheshire, UK) for use in a series of validation experiments. The kit is made up of fixative and eosin and methylene blue solutions. Raji cells were used because they contain approximately 50 EBV genomes per cell and therefore a single captured cell can be reliably detected

using RQ-PCR for EBV. Raji cells were laser microdissected and their DNA extracted by incubating at 50°C for 1 hour in various lysis buffers (see Appendix) followed by heat inactivation at 95°C for 10 minutes. Choice of lysis buffer was dependent on the downstream analysis. Lysates were assayed by RQ-PCR using a set of EBV *Bam*H1-W primers and probe (see Table 2.2). Viral sequences from 9 out of ten singly dissected Raji cells were amplified (see Figure 2.1), indicating the reliability of our LMD protocol.

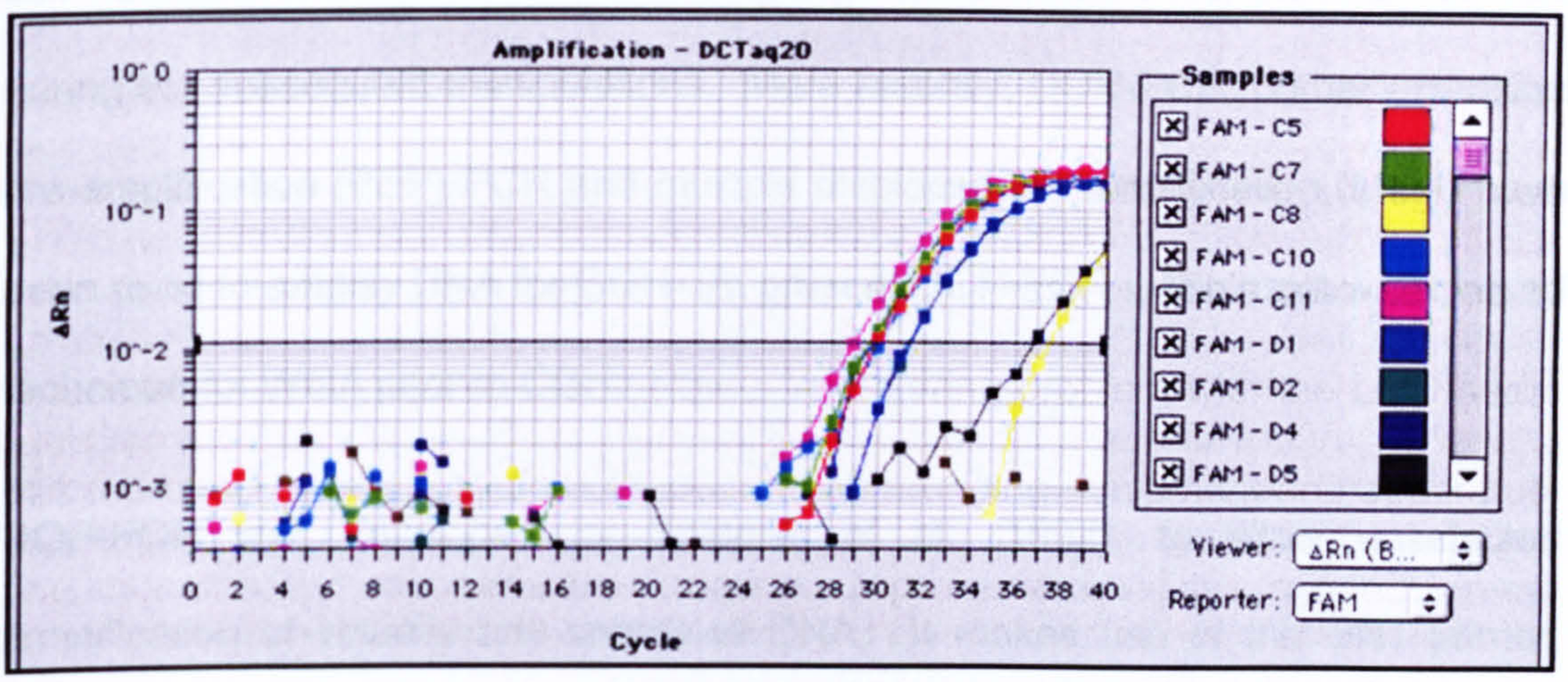

Figure 2.1 RQ-PCR amplification plot of EBV *Bam*H1-W detection following LMD of 10 singly dissected Raji cells. Each amplification plot represents a single cell. Nine out of the ten amplification plots exceeded the threshold ΔRn value.

2.8 Degenerate oligonucleotide primed PCR

The amount of DNA from 50 to 100 HRS cells obtained by LMD is too low for use in CGH. A single CGH experiment will need 1 to 1.5 μg of DNA. It is therefore necessary to use an amplification step to generate a sufficiently large quantity of genomic DNA, a procedure called whole genome amplification (WGA). Various

PCR-based and non-PCR based WGA techniques have been described in the literature. Early techniques (for example, linker adaptor PCR) involved ligation of specific sequences to each end of restriction enzyme digested DNA fragments followed by PCR using the adaptors as primers. Such approaches involved considerable manipulation of the sample before amplification including isolation of double-stranded DNA, restriction enzyme digestion and ligation. These techniques were unlikely to be useful when starting with a single cell or very small cell numbers since portions of the single genome would most probably be lost during the subsequent manipulations. More recently, DOP-PCR, primer extension pre-amplification (PEP) PCR and multiple displacement amplification (MDA) have been used to amplify DNA for CGH. At present DOP-PCR is the most widely used technique for WGA prior to CGH.

DOP-PCR was developed by Telenius et al. (1992), to allow unselected amplification of virtually any source of DNA. It makes use of the UN1 primer, which is a 22-mer containing 6 degenerate nucleotides (5'-CCG ACT CGA GNN NNN NAT GTG G-3'), and a mixture of low and high stringency PCR conditions. The primer was designed to give an optimal balance in terms of representation and yield when binding to genomic DNA. This technique has been specifically applied to chromosome painting, and results in a more uniform signal than ligation-based methods of WGA. In a PCR tube several low temperature annealings followed by extensions are performed to allow the primer to bind to multiple sites in the human genome. After several cycles of amplification at low annealing temperature, the annealing temperature is increased to allow more specific priming

only of fragments now tagged with the UN1 primer sequence. As much as 23,000-fold effective amplification can be achieved from as little as 15-26 pg of genomic DNA (Telenius et al. 1992). It has been estimated, based on the amount and sizes of DOP-PCR products, that there should be about one million DOP-PCR fragments generated from the entire human genome (Telenius et al. 1992). Since the average size of product from a DOP-PCR is 500 bp and the haploid human genome is about $3x10^9$ bp, it was estimated that an arbitrary stretch of DNA had only a one in six chance of being included in the DOP-PCR product. However, using a slightly modified DOP-PCR technique to amplify human DNA, Cheung et al. (1996) observed that all PCR-based markers tested were amplified and were accurately genotyped, suggesting the coverage is more comprehensive than the estimates (Cheung and Nelson 1996).

DOP-PCR was further optimised by Kuukasjarvi et al. (1997) using a new thermostable sequenase (ThermoSequenase) (Amersham, Buckinghamshire, UK) and low stringency conditions in the first 4 rounds of pre-amplification followed by amplification using AmpliTaq polymerase LD (Applied Biosystems) under more stringent conditions (Kuukasjarvi et al. 1997). Later, Huang et al. (2000) developed the 'improved DOP-PCR' protocol because they found the above 'optimised DOP-PCR' protocol could not generate reproducible and reliable results in their laboratory (Huang et al. 2000). They substituted the 10x high salt buffer, 10x low salt buffer, and AmpliTaq polymerase LD (Applied Biosystems) with ThermoSequenase buffer (Amersham, Buckinghamshire, UK), 10x AmpliTaq buffer and AmpliTaq polymerase (Applied Biosystems), respectively. The number

of cycles in the second round of DOP-PCR was increased to 35. These modifications made efficient amplification possible even when the amount of DNA template was reduced to 12.5 pg, with the resultant products suitable for CGH.

2.8.1 Optimisation experiments for DOP-PCR

Optimisation experiments were performed to determine: the DOP protocol that worked best in our laboratory; the best method of purifying primers, since primer purity has been found to be critical when amplifying small amounts of template DNA; the best method of cell lysis; and the optimal strategy for cutting cells for DOP-PCR.

Three DOP-PCR protocols were compared to determine sensitivity and efficiency. It was not possible to directly test the products by labelling and going through the whole process of CGH, as CGH had to be carried out in a distant laboratory. Gel electrophoresis and RQ-PCR were used to compare the amount of DNA generated and the representativeness of the products using the three protocols. Experiment to determine representativeness of the Huang's protocol is displayed in Figure 2.2. The primers and probe sets used in the RQ-PCR analysis are listed in Table 2.2.

Gene	3' primer sequence	5' primer sequence	Probe sequence
B-globin	GGCAACCCTAAGGT GAAGGC	GGTGAGCCAGGCC ATCACTA	CATGGCAAGAAAGT GCTCGGTGCCT
HSP 90	TGGCTGGTGACAGG AA	AAGGGCCGCAAGGT CTTC	AAACAGGCAAAGGC GCAGTCGC
AMPD	CCCTTCCATTGCCT CAGTTC	GGTAGCGGAAGTG GTTGCA	CATGAACACCACCT CACTAGTCTTCTGC CA
EBV Pol	AGTCCTTCTTGGCT AGTCTGTTGAC	CTTTGGCGCGGATC CTC	CATCAAGAAGCTGC TGGCGGCC
EBV LMP1	TCTAAGAAGCCACC ATGCGA	TGAAGGAACGGCG GAGAGTA	CGTAGAATCCAGCC AGTGGTCTACCCG
EBV *Bam*H1 W	CCCCTGGTATAAAG TGGTCCTG	CCCTCTTACATTTGT GTGGACTCC	AGCTATTTCTGGTC GCATCAGAGCGC
EBV EBER	AGGACCTACGCTGC CCTAGAG	AACCACAGACACCG TCCTCAC	AGCCACACACGTCT CCTCCCTAGCAAA
EBV ori P	AGGCGCAAGTGTGT GTAATTTGT	GGGCGGGCCAAGA TAGG	CTCCAGATCGCAGC AATCGCGC

Table 2.2 RQ-PCR probes and primers used in the optimisation of LMD and DOP-PCR.

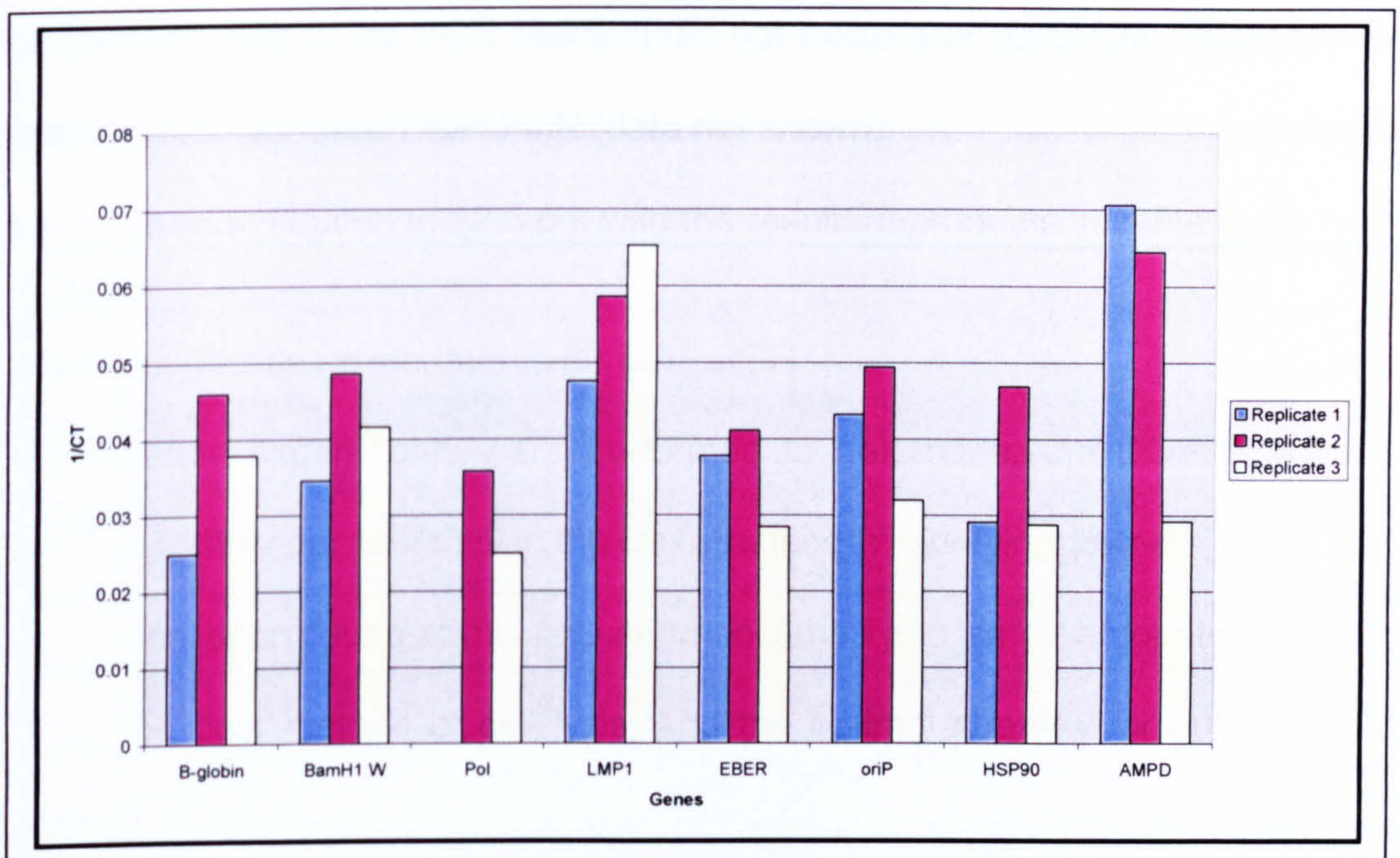

Figure 2.2 Representativeness and reproducibility of DOP-PCR as assessed by RQ-PCR using eight primer probe sets. This experiment was performed with 36 pg of starting DNA using the Huang's protocol. Standard curves have been generated for each experiment (data not shown).

Three methods of primer purification were compared: NAP, COP and HPLC purification. Slightly better amplification and consistency were achieved by using the NAP purified UN1-primer in comparison to HPLC or COP purified UN1-primer. The 'improved DOP-PCR' protocol of Huang et al. (2000) was more reproducible than either the original or optimised DOP-PCR protocol. The improved DOP-PCR protocol generated a large quantity of whole genome DNA from just 36 pg of starting DNA as assessed following 1% polyacrylamide gel electrophoresis (data not shown). The kinetics of the improved DOP-PCR protocol were assessed by the use of RQ-PCR (Figure 2.3). Increasing the number of cycles from 4 to 5 to 6

in the first step of the PCR reaction did not make any significant difference to the yield of this improved DOP-PCR (data not shown).

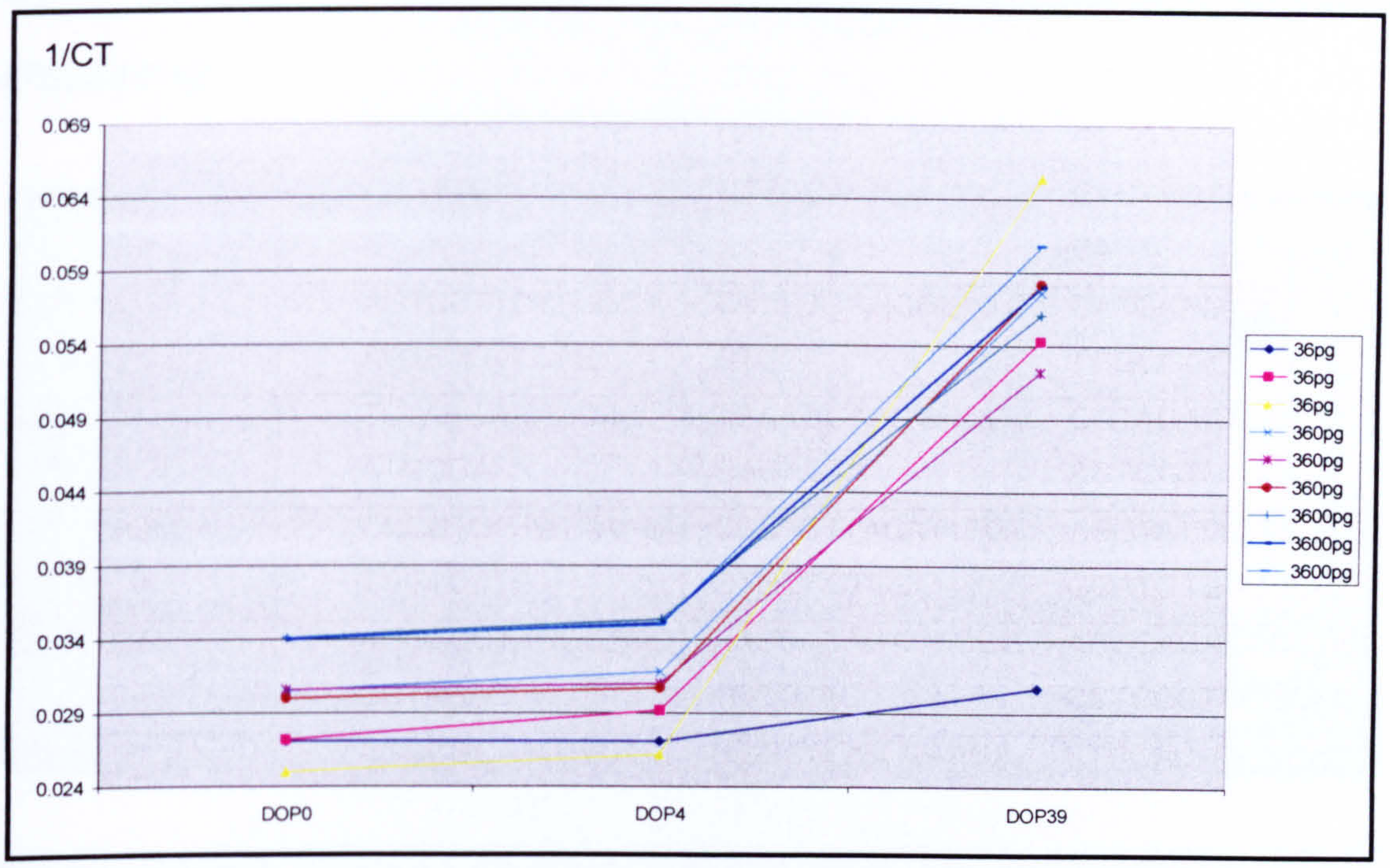

Figure 2.3 Copy number changes in β-globin gene as quantified by RQ-PCR using differing amounts of starting KMH2 DNA. Samples were analysed following LMD (DOP O), following 4 cycles of DOP amplification (DOP 4) and following 39 cycles of amplification.

Varying the temperature (50°C or 55°C) or duration (1 hour or 2 hours) of incubation at the point of proteinase K digestion seemed to make little difference to the success of the improved DOP-PCR protocol, whereas the addition of a non-ionic detergent, such as polyoxyethylenesorbitan monolaurate (Tween 20) (Sigma) and NP40, to the lysis buffer resulted in better amplification. Using the above conditions, the efficiency of different laser microdissection strategies followed by DOP-PCR was compared with analysis using RQ-PCR and the EBV *Bam*H1 W

assay. The best approach was to cut 5 aliquots of 10 Raji cells, amplify them with the improved DOP-PCR individually and then pool them together, rather than amplify 50 cells in a single tube. This strategy gave the most consistent yield (Figure 2.4).

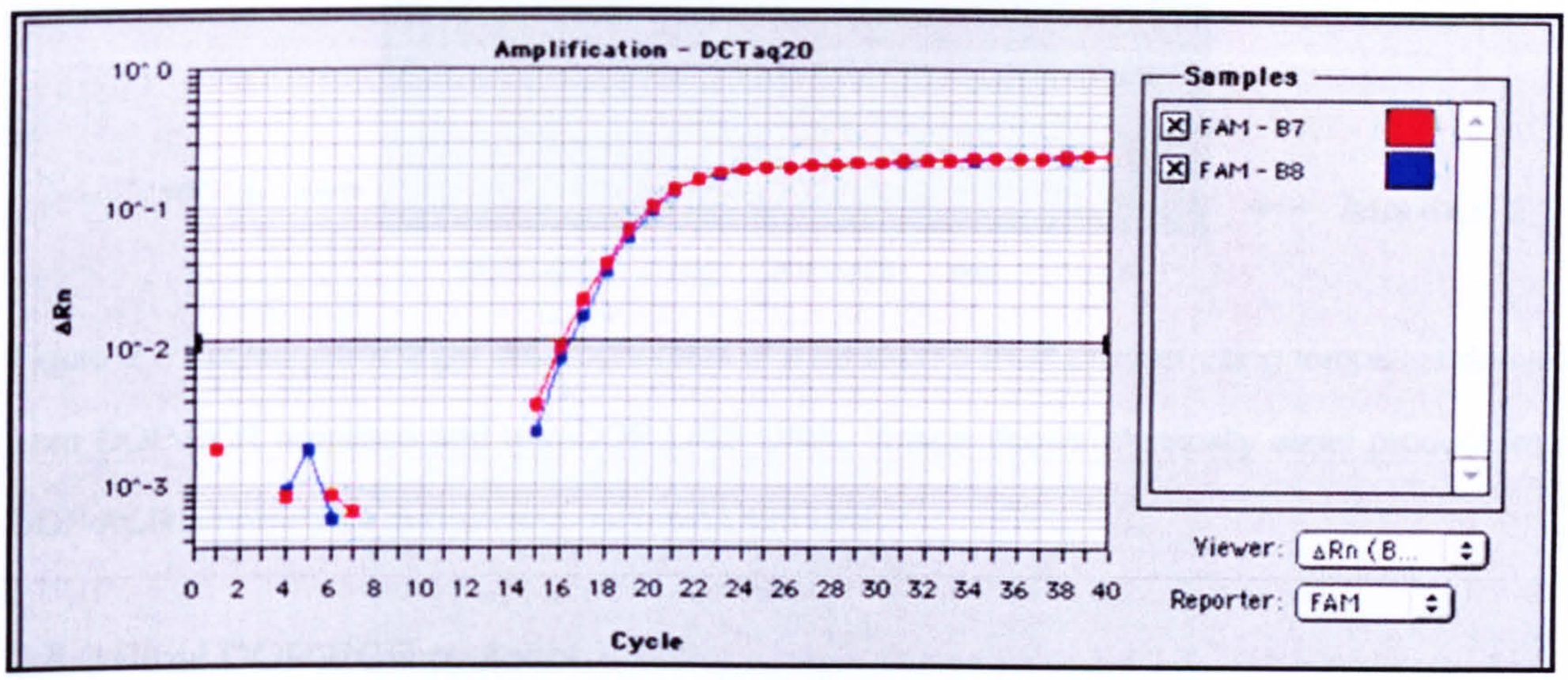

Figure 2.4 RQ-PCR amplification plot of EBV *Bam*H1 W detected from pooled DOP-PCR amplified products derived from 5 aliquots of 10 laser microdissected Raji cells. Two replicates showing good reproducibility with overlapping plots.

2.8.2 Fidelity of 'improved DOP-PCR'

In order to assess the fidelity of the DOP-PCR, the presence of a mutation in the PCR (refer to *IkBa* PCR) was performed using either DOP-amplified products from L428 cell line was investigated. A single base mutation of C to T at position 2278 in *IkBa* exon 5 was reliably detected from DOP-PCR amplified L428 DNA. Triplicates of *IkBa* PCR products were visualised by 1% polyacrylamide gel electrophoresis. All samples tested revealed a PCR product size band similar to positive controls and direct sequencing of the DOP-PCR products confirmed the

presence of the C to T change as previously described in genomic DNA from L428 (Figure 2.5). This experiment confirmed the fidelity of this improved DOP-PCR protocol.

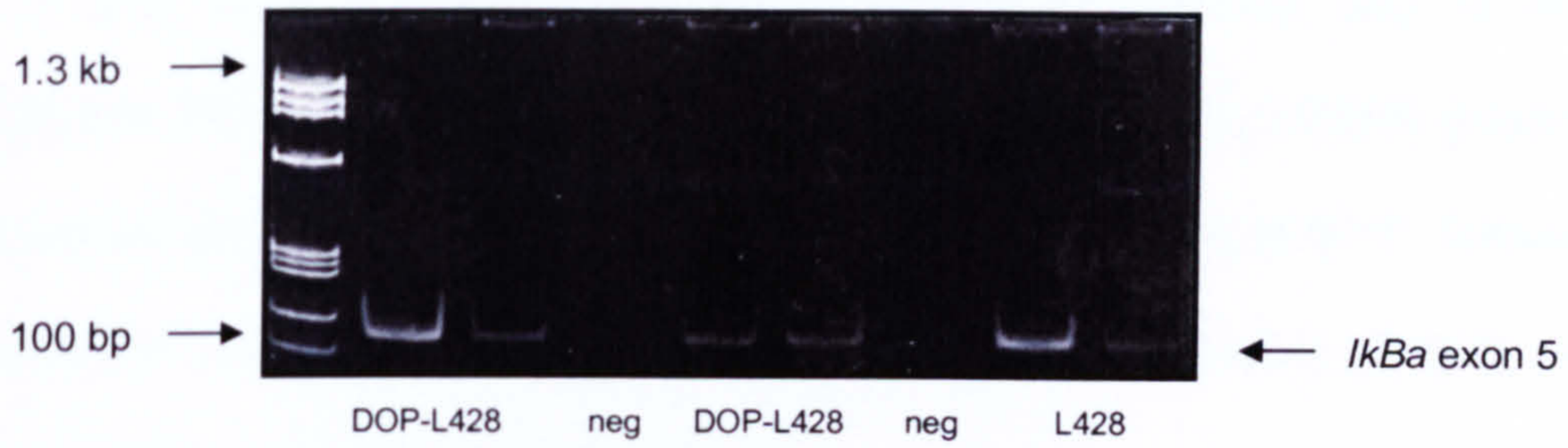

Figure 2.5 Polyacrylamide gel electrophoresis of *IkBa* exon 5 PCR product using templates derived from DOP-PCR amplified and non-DOP L428 DNA. Image shows identically sized product from DOP-PCR amplified L428 DNA and genomic L428 DNA.

2.8.3 Final DOP-PCR protocol

In the experiments described in this thesis, 5 x 10 laser microdissected cells were lysed in 6 μl of ThermoSequenase buffer containing non-ionic detergent and proteinase K (see Appendix) by incubation at 55°C for 1 hour. Proteinase K was subsequently inactivated by incubation at 95°C for 10 minutes. DOP-PCR was performed using a GeneAmp PCR System 2400 or 9700 (Applied Biosystems) in two steps according to the 'improved DOP-PCR' protocol (Huang et al. 2000). In step I, DNA was amplified in a 10 μl reaction volume containing 200 μM of each dNTP, 1 μM UNI-primer (Telenius et al. 1992), 4 units of ThermoSequenase DNA polymerase (Amersham), and 1x ThermoSequenase reaction buffer (26 mM Tris-HCl, pH 9.5; 6.5 mM $MgCl_2$). Thermal cyling conditions consisted of 3 minutes at 95°C, followed by 4 cycles of 1 minute at 94°C, 1 minute at 25°C, 3 minutes

transition from 25-74°C, 2 minutes extension at 74°C, and a final extension of 10 minutes at 74°C. In step II, the reaction volume was increased to 50 μl by the addition of 40 μl of a mastermix containing 160 μM of each dNTP, 1.2 μM UNI-primer, 5 units AmpliTaq DNA polymerase (Applied Biosystems), and 1x PCR buffer (10 mM Tris-HCl, pH 8.4; 50 mM KCl; 1.5 mM $MgCl_2$; and 0.001% gelatine; Applied Biosystems). Thermal cycling conditions were 3 minutes at 95°C, followed by 35 cycles of 1 min at 94°C, 1 minute at 56°C, 2 minutes extension at 72°C, and a final extension of 10 minutes (Figure 2.6).

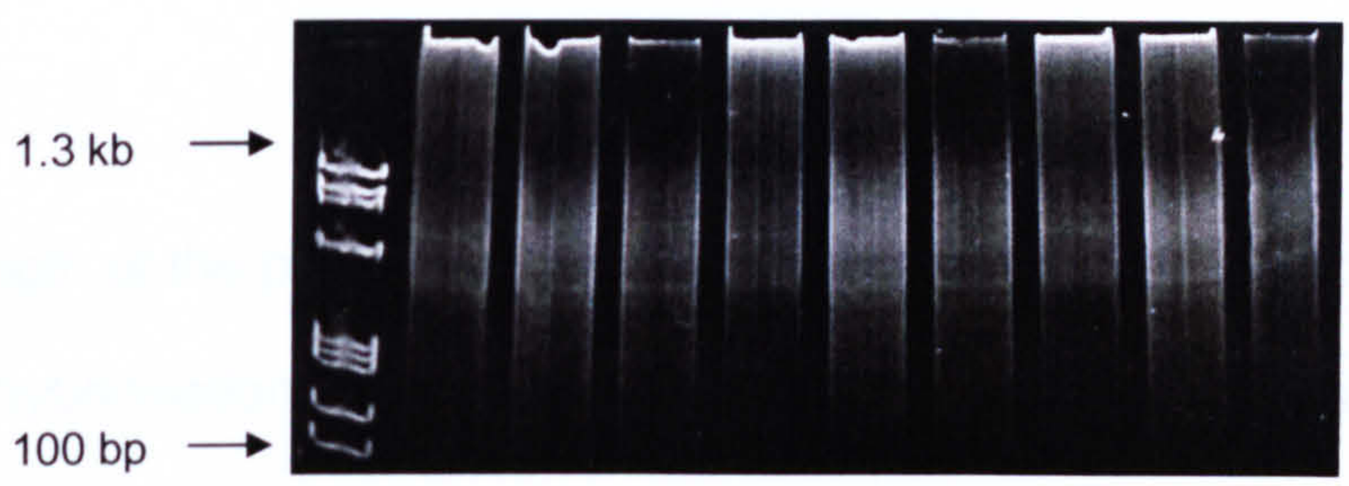

Figure 2.6 Agarose gel electrophoresis of identical replicates of DOP-PCR products using 60 pg DNA as starting material.

2.9 Outline of CGH

The following sections will describe the main steps involved in CGH once an adequate amount of DNA template is generated; namely labelling with fluorochromes, hybridisation on metaphase slides, image capturing and analysis.

2.9.1 Labelling of PCR products by nick translation

DNA probes for use in CGH can be labelled either directly or indirectly. Although directly labelled probes generate smooth fluorescence along the length of the chromosomes, indirect detection procedures also result in good quality CGH

results. Indirect labelling results in signal amplification but can lead to a greater background. Labelling of DNA can be achieved by random priming, nick translation or during DOP-PCR, utilising either biotinylated deoxynucleotides or deoxynucleotides conjugated with digoxigen or fluorochromes. For many applications using high molecular weight DNA, nick translation is the method of choice. Nick translation is more suitable for this application because probe length can be controlled by enzyme titration. Commonly used fluorochromes for test and control DNA are FITC and rhodamine. SpectrumRed-dUTP and SpectrumGreen-dUTP (Abbott Laboratories, Berkshire, UK) were used in labelling reactions in this project.

The length of the probe molecules after labelling is a critical factor for good quality *in situ* hybridisation. The optimal fragment size range of labelled DNA to obtain a homogenous hybridisation pattern is 300 to 2000 bp. This is longer than the probe length used in other types of FISH applications. Longer fragments improve both the intensity and uniformity of the signals obtained. During nick translation, the exonuclease of DNA polymerase causes a single-strand break (or nick) in the DNA. Nucleotides (both labelled and non-labelled) are added to the 3' end of the nicked strand by DNA polymerase, using the DNA sequence of the non-nicked strand as template. During nick translation the length of the fragments may be modified by altering the ratio of DNase I to DNA polymerase I enzymes in the nick translation reaction mixture. In CGH it is important to adjust not only the length of the fragments, but also to achieve a similar length for test and control DNA (Larsen et al. 2001).

In a 1.5 ml microfuge tube, the reagents were added in the following order: approximately 1.5 μg of DNA, mixture of unlabelled nucleotides at 100 mM, labelled nucleotides at 1 mM, water to make up to 50 μl, optimal amount of DNA polymerase 1 (0.4 U/μl) and DNase 1 (40 pg/μl). Reagents were mixed thoroughly and were pulse-centrifuged in a microfuge. The reaction mixtures were incubated at 15°C for 45 minutes in the case of DOP-PCR products and 1 hour 40 minutes in the case of genomic DNA samples. Reactions were terminated by incubating at 70°C for 10 minutes.

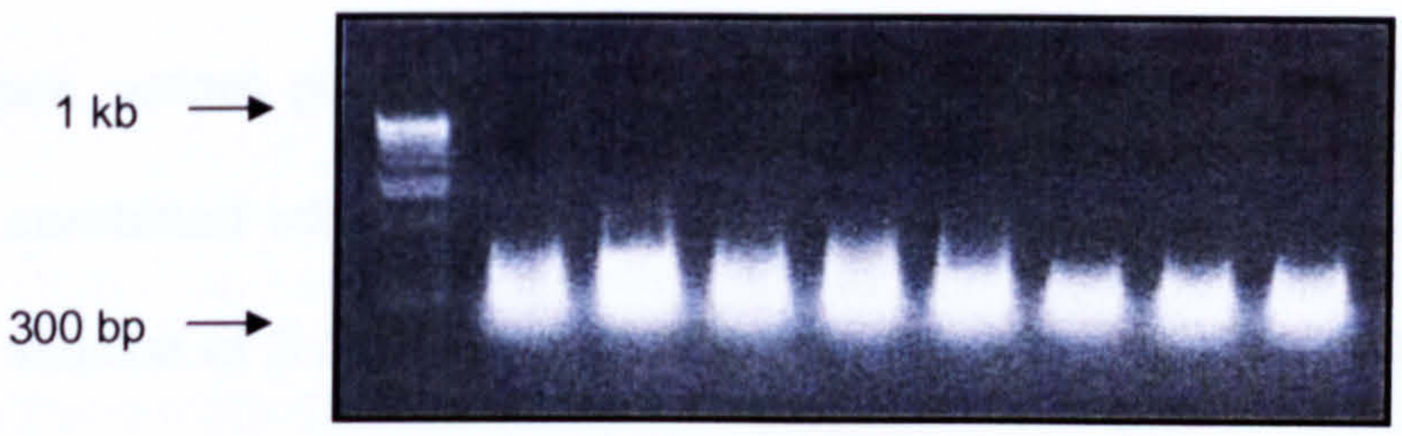

Figure 2.7 Agarose gel electrophoresis of identical replicates of SpectrumGreen labelled DOP-PCR products from laser microdissected HRS cells (5 x 10 cells).

In order to increase fragment size the amount of DNA polymerase 1 was increased and/or the amount of DNA polymerase 1/DNase 1 mix or incubation time was decreased. The product size range was checked by running approximately one fifth of the reaction on a 1% agarose gel (Figure 2.7). Labelled probes were stored in the dark at -20°C.

2.9.2 Slide preparation for CGH

One of the most critical parameters for successful CGH is the quality of metaphase spreads. Pretreatment of the specimen by proteolytic digestion can improve the

accessibility of probes to the specimen. However, such a treatment may also result in a higher granularity of the signal generated by the genomic DNA, thus hampering the CGH analysis. In these experiments, metaphase spreads were purchased from a commercial supplier (Abbott Laboratories), in order to circumvent problems associated with quality assurance. The method for making normal metaphase spreads is described in section 2.10.7.1.

2.9.3 Preparation of probe mix

The labelled DNA was light sensitive therefore manipulations were performed in the dark, where possible. Approximately 800 – 1000 ng of each labelled DNA were combined with 60 μg of human CoT-1 DNA (Invitrogen, Paisley, UK). One tenth volume of 3 M sodium acetate and 2.5 times volume of 100% ethanol were added to precipitate the DNA. The mixture was vortexed briefly and placed in the dark at -80°C for 30 minutes. The mixture was centrifuged at maximum speed (13000 rpm) in a microfuge at 4°C for 30 minutes. The supernatant was discarded and the pellet washed briefly with 100 μl of 70% ethanol and centrifuged at maximum speed (13000 rpm) in a microfuge at RT for 1 minute. All supernatant was removed and the pellet was dried in the dark without heating in a SpeedVac (ThermoSavant) for 10 minutes at RT. Once dried, the pellet was resuspended in 3 μl dH_2O and 7 μl CGH hybridisation buffer (Abbott Laboratories). The probe mix was kept on ice and then equilibrated to RT immediately prior to use.

2.9.4 Hybridisation of the probe to target metaphase spreads

The denaturation solution (see Appendix) was warmed from cold to the desired temperature (usually 73°C but 74°C for older slides) in a waterbath. Target metaphase slides (Abbott Laboratories) were taken from storage at -20°C and the area containing the metaphase spreads was marked with a diamond scribe. The slides were immersed in denaturation solution for 5 minutes and, when 3 minutes were remaining, the probe mix was denatured by incubating at 73°C on a heat block for 5 minutes. The slides were dehydrated through an ethanol series (70%, 85% and 100%) for 1 minute each and allowed to dry. Following denaturation, the probe mix was left at RT in the dark for 1 minute to self-anneal. The entire probe mix (10 µl) was then applied onto the target area which was then covered with an 18 x 18 mm 'Thickness 1' coverslip (BDH) and sealed with rubber cement. The slides were sealed in a humidified box and incubated in the dark at 37°C for 4 days.

2.9.5 Post-hybridisation washes

Wash Buffer 1 (see Appendix) was warmed slowly from RT to 74°C. The rubber cement and coverslips were gently removed from the slides, which were then immersed in Wash Buffer 1. The slides were agitated for a few seconds and then left for 2 minutes. It was not advisable to wash more than 4 slides at once because this caused a drop in the temperature of the buffer. The slides were then transferred to Wash Buffer 2 (see Appendix) at RT, agitated for a few seconds and left for 1 minute. Excess buffer solution was removed by blotting the end of the

slide on paper towels and standing upright for a few seconds in the dark. DAPI II (Abbott Laboratories) counterstain (12.5 μl) was added to each area of hybridisation, and a 22 x 55 mm 'Thickness 1' coverslip was applied. Excess liquid was removed from around the coverslip with a tissue and the slides were sealed with clear nail vanish. The slides were kept at 4°C in the dark until image capturing.

2.9.6 Hardware requirements for CGH

Visualisation of fluorescent signals was performed with a Zeiss Axioskop fluorescence microscope (Carl Zeiss, UK) equipped with fluorescence filters for DAPI, FITC and TRITC and a double or triple band pass filter. The selection of appropriate filter sets was critical since crosstalk between different fluorochromes, which will bias CGH ratio measurements, can occur. A relative lateral displacement of images in different colours can also be caused by a mechanical imperfection of the filter sets. Optical shift becomes negligible if either a computer-controlled filter wheel or microscopes with an automatic filter change are used for image acquisition.

2.9.7 Image capturing

CGH images were captured by a CCD camera (Photometrics, Ottobrunn, Germany) with a high sensitivity, high spatial resolution, high dynamic range and low noise. Image capturing was carried out in the dark. Immersion oil was applied over the hybridisation area and metaphases were screened using the DAPI filter at x 630 magnification. Metaphases were selected based on a number of criteria,

including smooth hybridisation signals across each chromosome, low non-specific background hybridisation, low binding of the labelled probes at the centromeres and heterochromatic regions, and adequate DAPI banding patterns for later chromosome identification. Good quality metaphases, i.e., those with well-stretched but not over stretched chromosomes and with fewer than 2 crossover chromosomes, were selected. The IPLAB software (Abbott Laboratories) was used to capture at least 5 and up to 15 quality metaphases. It was important to make sure that the camera was in focus prior to capturing each metaphase. Images were all saved as PICT files.

2.9.8 Image analysis

Chromosomal gains and losses were assessed on the basis of differences in signal intensities generated by dye-labelled test and control DNA along the chromosomes. Although many differences can be detected by visual inspection, the quantitative assessment of the ratio of both fluorochromes by digital image analysis gives higher accuracy and greater resolution. The theoretical ratio of the signals from the test and control genomes is: 1 (following normalization of fluorescence intensities) in cases where the chromosomal region is balanced; 0.5 where there is a deletion, monosomy, or hemizygosity; and 1.5 where there is partial or complete trisomy, etc. For higher degree amplifications, much higher ratios are anticipated. Generally accepted criteria for CGH evaluation by digital image analysis are a matter for discussion. Fixed thresholds do not take into account the variation of ratio values between different experiments, different

metaphases from the same slide and even between different chromosomal regions.

Images were analysed with Quips CGH software (Abbott Laboratories). PICT files were first converted to ICS files, which were read by the Quips karyotyping software. Each metaphase image had its contrast adjusted, chromosomes trimmed and cut tightly and then the chromosomes were arranged into their correct order. Karyotypes were derived from DAPI images and the chromosomes were automatically straightened. The axis was determined and profiles for all colours were computed by summing up the intensity values along lines orthogonal to the axis. The ratio values were automatically normalised in such a way that normal chromosomes have a mean ratio value of 1. For a single CGH analysis, 10-15 homologues of each chromosome were measured after DAPI karyotyping of 5-10 metaphases. Average ratio profiles were then calculated after automatically scaling the profiles of individual homologous chromosomes of the same length. The average profiles were displayed side by side with ideograms of all chromosomes.

Caution was exercised in evaluating the final data from a number of specific chromosomal regions. Pericentromeric and heterochromatic regions could not be reliably assessed as they were blocked by unlabelled CoT-1 DNA. Since these regions are polymorphic between individuals, the extent of blocking is highly variable and these regions were therefore excluded from the analysis. As the fluorescence intensity decreases towards the telomere the signal approaches

background. Fluorescence ratios from telomeric regions were therefore unreliable and these regions were generally excluded from analysis.

To accurately assess chromosomal imbalances by variations in the fluorescence intensity ratios, suppression must be as complete as possible. An excess of unlabeled human CoT-1 DNA was therefore used and the pre-annealing time elongated. With a pre-annealing time of 15 minutes, 1 μg of test DNA and 1 μg of control DNA were combined with 40 to 70 μg of CoT-1 DNA, adjusted to 0.15 M sodium acetate, and co-precipitated by adding 2 volume of cold ethanol. The large amount of DNA also required an extensive agitation during resuspension of the precipitated probes in formamide. The quality of CGH experiments was also improved by extension of the *in situ* hybridisation time from overnight to 2 or 3 days (Lichter et al. 1995).

2.10 FISH and FICTION

FISH and FICTION were used in the analysis of imprints, cytospins and sections of paraffin-embedded material.

2.10.1 FISH probes

FISH probes were made from labelled bacterial artificial chromosome (BAC) clones. BAC clones used in this project were obtained from the BAC library of the Roswell Park Cancer Institute (RPCI-11) (http://bacpac.med.buffalo.edu). Additional clones were purchased from the German Resource Centre for Genome Research (http://rzpd.de) or kindly provided by the Sanger Centre (http://www.sanger.ac.uk).

2.10.2 Design of FISH probes

One of the goals of the Human Genome Project (HGP) was to construct detailed genetic and physical maps of the human genome (Lander et al. 2001). To accomplish such a purpose, DNA was fragmented and inserted into cloning vectors, commonly BACs or P1-derived artificial chromosomes (PAC). These clones are between 100 and 300 kb in size, which makes them suitable for FISH experiments. Sequence data are available online through several centres involved in the HGP. Suitable clones were identified by searching the genome sequence database using the basic local alignment search tool (BLAST) algorithm at the National Centre for Biotechnology Information (NCBI) server (http://www.ncbi.nlm.nih.gov). To obtain further information about overlapping and contiguous clones, the mapping resources provided online by Project Ensembl (http://www.ensembl.org) were used. The Ensembl Genome Browser was a joint project, between EMBL-EBI and the Sanger Institute, to develop software which is freely available to all scientists.

2.10.3 Isolation of BAC DNA

DNA was extracted by using the Perfectprep Plasmid Maxi Kit (Eppendorf, Cologne, Germany) according to the instructions of the manufacturer. Bacteria containing BACs were grown overnight in 150 ml of LB medium (see Appendix) supplemented with the appropriate antibiotics. After DNA preparation, a final concentration of 50-200 ng/μl of high-purity BAC DNA in a volume of 200 μl sterile water was obtained. Quantification of DNA was performed as described in section 2.5.

2.10.4 Labelling of BAC DNA

Probe labeling was performed using the random priming method (Bioprime, Gibco/Life Technologies, Eggenstein, Germany) exchanging the dNTP-Bio with a dNTP mixture containing the appropriate fluorochrome. Two dUTP conjugated fluorophores, SpectrumOrange (SO) and SpectrumGreen (SG) (Abbott Laboratories), were used in combination with dTTP for the labeling reactions. A 10x dNTP mixture was created by mixing dATP, dCTP, dGTP, dTTP and dUTP-fluorophores at a final concentration of 1 mM, 1 mM, 1 mM, 0.5 mM and 0.5 mM respectively.

One microgram of DNA was diluted with dH_2O to a final volume of 24 µl. Twenty microlitres of 2.5x random octamers were added to the DNA solution which was then denatured by heating in boiling water for 5 minutes. The mixture was immediately cooled on ice. Five microlitres of dNTP mixture containing SO/SG-dUTP and 1 µl of Klenow fragment were mixed gently but thoroughly with the DNA solution. The entire mixture was incubated at 37°C overnight with 5 µl of Stop Buffer (Bioprime, Gibco/Life Technologies, Eggenstein, Germany) added the next day to terminate the reaction.

2.10.5 Probe purification: Sephadex G50

A small amount of sialinized glass wool was inserted up to the 0.2 ml mark of a 1 ml syringe and Sephadex G50 solution (see Appendix) added up to the 1 ml mark. The syringe was placed in a 15 ml centrifuge tube and subjected to centrifugation at 2000 g for 10 minutes at RT (Centrifuge J-6B Beckman, High Wycombe, UK).

The liquid that passed through the syringe was discarded and Sephadex G-50 resin was added again to the 1 ml mark and centrifuged as above. This step was repeated until the resin was tightly packed up to the 1 ml mark. The column was then washed 3 times by adding 100 µl of column buffer (see Appendix) and spinning at 2000 g for 10 minutes. An uncapped 1.5 µl microfuge tube was placed inside the 15 ml centrifuge tube beneath the syringe. The labelled DNA probe solution was loaded onto the column and centrifuged as before. The purified DNA probe was collected and stored in the dark at 4°C for 2 months or at -20°C for longer time periods.

2.10.6 Probe preparation for hybridisation

Ten microlitres of each probe (approximately 200 ng) were added to 5 µl of CoT-1 DNA (1 mg/ml) followed by 1/10 volume of 3 M sodium acetate at pH 5.2. Two and a half volumes of 100% ethanol were added and the mixture was inverted several times. The probe was precipitated by centrifugation at full speed (17000 rpm) for 30 minutes at RT (Biofuge 22R, Heraeus, Osterode, Germany). The supernatant was decanted and as much ethanol as possible was removed with a pipette. The pellet was dried in the dark for approximately 20 minutes. Ten microlitres of 50% hybridisation master mix (see Appendix) were then added to the dried probe pellet. If a commercial probe had to be added to a non-commercial probe, 1 µl of the commercial probe was added at this stage with thorough mixing. The probe was resuspended by constant shaking at RT for 1 hour. Quality of resuspension was checked by placing the end of the tube over an UV transilluminator.

2.10.7.1 Metaphase slide preparation

Normal cell suspensions left over from conventional cytogenetic analysis were used. In a humidity chamber, normal metaphase slides were prepared by dropping two or three drops of cell suspension onto a clean glass slide held at an angle with a Pasteur pipette. The slides were then left to dry for 10 minutes. A diamond scribe was used to circle the areas with good metaphase spreads, which were selected under a phase contrast microscope.

2.10.7.2 Slide preparation for FICTION

Lymph node imprints and cytospins were used for FICTION studies. Acetone fixed slides were frozen at –80°C until use. Cytospin slides were prepared from viable cell suspensions with a cell count of approximately $2.5x10^5$ cells per spot. Cell suspensions (100 µl per chamber) were spun in a cytocentrifuge (Cytospin 2, ThermoShandon) for 10 minutes at 450 rpm to achieve a circular imprint of 0.5 cm diameter. It was important to get an even monolayer of cells. If the cells appeared clustered or overlapped, immunophenotyping and FISH at the single cell level were impossible.

2.10.8 Fluorescence immunophenotyping for FICTION

Coverplate™ technology from ThermoShandon (Frankfurt, Germany) was used to aid immunophenotyping of the HRS cells for FICTION. This system uses plastic coverplates where the slide could be securely attached leaving just a tiny gap between the plate and the slide. The system can hold 100 µl of reagent vertical

which is maintained in even contact with the cells. It has the advantage of keeping the slides in the dark throughout the staining procedure and minimising the use of reagents. Ten slides and coverplates were fixed in a special slide rack assembly. PN buffer (see Appendix) was pipetted into the space between the slide and plastic coverplate until just 100 µl was retained by capillary action. The addition of antibody will then displace the PN buffer from the coverplate. It was important not to use any detergent in the buffers as these reduce surface tension and the reagent will no longer be trapped by capillary action. The use of an ImmunoPen to circle cell spots will also prevent good contact between the reagents and cells.

The cells were washed once in PN buffer and 100 µl of BerH2 primary antibody diluted 1:20 in PNM (see Appendix) was added. Slides were incubated at RT for 30 minutes or overnight at 4°C. Overnight incubation was useful in obtaining stronger staining intensity without significantly increasing background staining. The cells were then washed once in PN buffer at RT. One hundred microlitres of Alexa594-conjugated rabbit anti-mouse antibody (1:50 diluted in PNM buffer) were added and the preparations were incubated at RT for 30 minutes. The cells were then washed in PN buffer. The cytospins were detached from the Shandon holders and were placed in a Coplin jar containing PN buffer. The slides were mounted in PN buffer and the quality of ICC was checked briefly under a fluorescence microscope. The best areas were selected by drawing a circle with a diamond scribe in the region with optimal density of positive cells.

2.10.9 1 Pretreatment of normal metaphase slides

Slides were incubated in freshly prepared pepsin digestion solution (see Appendix) for 5 minutes at 37°C to remove cellular cytoplasm. The slides were washed in dH_2O for 1 minute followed by fixation in 1% paraformaldehyde for 2 minutes. The slides were washed once again in dH_20 for 1 minute and were dehydrated through an ethanol series of increasing percentage (70%, 85% and 100%) for 2 minutes each. The slides were air-dried at room temperature for 10 minutes.

2.10.9.2 Pretreatment of slides for FICTION

The coverslips were carefully removed and the cells were fixed in fresh Carnoy's fixative (Methanol:acetic acid 3:1) for a minimum of 10 minutes. The cells were washed once in dH_20 for 1 minute and then fixed in 1% paraformaldehyde for 1 minute. Following further washing, preparations were dehydrated through an ethanol series of increasing percentage (70%, 85% and 100%) for 2 minutes each. The slides were allowed to dry in the dark for 20 minutes. It was important to minimise light exposure to prevent fading of immunofluorescence.

2.10.10 Simultaneous denaturation and hybridisation

The probe mixture (1.3 – 1.5 µl) was applied onto a small (10 mm) round coverslip and the cellular area was gently lowered onto the coverslip. The coverslip was sealed with rubber cement and the slides were placed inside a humid metal box which was placed in a waterbath previously heated to 70°C. Both probe and target DNA were denatured simultaneously for 7-12 minutes. Following denaturation, the humid metal box was placed in an incubator at 37°C overnight.

2.10.11.1 Post-hybridisation washes and slide mounting for FISH

Wash Buffer 1 (Abbott Laboratories) was pre-warmed in a water bath at 73°C for one hour. Both the rubber cement and coverslip were removed carefully and the slides were placed in Wash Buffer 1. The slides were shaken for a few seconds and were left in the wash buffer for 5 minutes. The slides were then transferred into Wash Buffer 2 at RT, shaken and washed for 5 minutes. A five minute incubation in DAPI solution was performed to counterstain the DNA. Finally the slides were washed in 2x SSC for 1 minute prior to mounting with antifade solution (see Appendix) and 'Thickness 1' (22 x 60 mm) coverslips.

2.10.11.2 Post-hybridisation washes and slide mounting for FICTION

Three Coplin jars of 0.1x SSC were placed in a waterbath at 60°C for one hour. The rubber cement was removed gently from the slides and the coverslip shaken off in the first Coplin jar of 0.1x SSC. The slides were left to wash for 5 minutes. This was followed by 2 subsequent washes in 0.1x SSC for 5 minutes each. The slides were washed in PN buffer for 1 minute at RT in the dark. DAPI solution was used to counterstain the cells by incubating for 5 minutes. The slides were finally washed in 2x SSC for 1 minute and were mounted with antifade solution (see Appendix) and 'Thickness 1' (22 x 60 mm) coverslips. Vectashield antifade (Vector Laboratories) was preferred when sections of paraffin-embedded material were used.

2.10.12 Digital image capturing

Slides were analysed by use of a Zeiss Axioskop2 fluorescence microscope (Göttingen, Germany) equipped with blue (DAPI), green (SpectrumGreen) and red (Texas Red) wide band-pass filters as well as double (green/red) and triple (blue/green/red) filters (AHF, Tübingen, Germany). In addition, the microscope had extra narrow band-pass filters specific for SpectrumBlue and SpectrumOrange/Gold. Images were documented using the ISIS 3.0 imaging system (MetaSystems, Altlussheim, Germany). The software was capable of transforming 3-dimensional information, such as signals at 2-3 focal planes, into a single 2-dimensional image.

2.10.13 Probe evaluation

The quality of FISH was evaluated by hybridising the labelled probe onto normal metaphase spreads from healthy donors. The main parameters to take into account when a new FISH probe was designed were: signal to noise ratio, location of the probe, chimerism and cross-hybridisation (Martin-Subero et al. 2003).

2.10.14 Evaluation of fluorescence immunophenotype

A problem in FICTION is the frequent autofluorescence from some cell types, particularly eosinophils, as well as the intercellular matrix components and sclerotic areas of sections. This autofluorescence was visible through blue, green and red filters, but not through the infrared filter. The resultant image captured would give a white matrix surrounding the cells and also within cells in some cases. This had to be taken into account when evaluating the immunophenotype

obtained using Alexa 594 (Invitrogen, Paisley, UK). Specific immunofluorescence signals had to be more intense than the non-specific antibody binding and clearly differentiated from autofluorescence. Different patterns of immunofluorescence were distinguished: nuclear, cytoplasmatic and membrane, although these could be hard to differentiate since the cells were intact, not sectioned. Thus, the nucleus might be overlaid with cytoplasm leading to 'pseudo-nuclear' staining.

2.10.15 Evaluation of cell morphology and quality of DNA

Both hybridisation signals and immunophenotyping had to be correlated with cell size and shape. Usually, positive ICC and aberrant hybridisation patterns were correlated to a given cell morphology within the tumour biopsy. In cases where the immunophenotyping appeared to be satisfactory but yet no FISH signals were found, the DAPI staining was invaluable. The DAPI staining of the nuclei was a good indicator of the quality of DNA and this correlated strongly with the success of FISH.

Chapter 3

RECURRENT GENOMIC IMBALANCES IN HODGKIN AND REED-STERNBERG CELLS

3.1 Introduction

Cytogenetic analysis has been extremely productive in the investigation of non-Hodgkin lymphoma (NHL) and leukaemia, where the identification of non-random chromosomal abnormalities has led to the discovery of numerous oncogenes (Rowley 1973; Pelicci et al. 1986; Rosenberg et al. 1991; Raimondi 1993; Tilly et al. 1994; Pulford et al. 1997). A conventional cytogenetic approach to the investigation of Hodgkin lymphoma (HL) has been hindered because of the small number and low mitotic index of Hodgkin and Reed Sternberg (HRS) cells.

Comparative genomic hybridisation (CGH) is an alternative approach to studying molecular cytogenetic abnormalities in combination with laser microdissection (LMD) and DOP-PCR (see chapter 2). This chapter will describe our analysis of 20 cases of classical Hodgkin lymphoma (cHL) using CGH. In this study HRS cells were isolated from cytospins on the basis of both CD30 ICC and morphological criteria, thus ensuring that pure populations of *bona fide* HRS cells were selected for further analysis.

3.2 Materials and methods

3.2.1 Clinical cases, cell lines and experimental design

Twenty cases of cHL from which viable material was available were selected. Cases included 9 male and 11 female patients, aged 14-84 years at the time of diagnosis (Table 3.1). All patients gave informed consent for their tissue to be used and this study was approved by a multicentre research ethics committee. Sections from all cases were reviewed by an expert lymphoma pathologist and

classified according to the WHO guidelines (Stein et al. 2001). EBV status was determined by EBER *in situ* hybridisation as previously described (Armstrong et al. 1998). Cases in which the HRS cells stained positively are referred to as EBV-positive cHL.

Case	Sex	Age (years)[a]	Subtype[b]	EBV status[c]	Outcome[d]
A	M	39	LR	+ve	A
B	F	46	MC	+ve	NA
C	F	46	NS	-ve	A
D	M	67	NS	-ve	A
E	M	27	NS	-ve	A
F	F	37	NS	-ve	NA
G	F	50	LR	+ve	D
H	M	33	NS	-ve	A
I	M	19	NS	-ve	R
J	F	16	NS	-ve	A
K	F	14	NS	-ve	A
L	M	21	NS	-ve	NA
M	M	19	MC	+ve	A
N	F	27	NS	-ve	NA
O	F	41	MC	-ve	A
P	M	55	NS	-ve	R
Q	F	37	NS	+ve	A
R	F	58	LD	+ve	R
S	M	31	NS	-ve	NA
T	F	84	MC	-ve	R

Table 3.1 Patient Details including age, sex, histological subtype, EBV status and clinical outcome.

([a]Age at diagnosis; [b]MC, mixed cellularity, NS, nodular sclerosis, LR, lymphocyte rich, LD, lymphocyte depleted; [c]Presence (+) or absence (-) of EBER staining in the HRS cells; [d]A, alive, R, relapsed, D, dead, NA, not available).

Validity testing of the DOP-PCR-CGH was performed using: the IM9 cell line, a lymphoblastoid cell line with a female, diploid, stable karyotype (ATCC, Manassas, VA, USA); Daudi, a BL cell line with a male, diploid, stable karyotype (ATCC); and MPE600 (Abbott Laboratories, UK), a breast cancer cell line with known CGH profile. DNA extracted from 4 HL-derived cell lines, namely L428, L1236, KM-H2 and L591, was also investigated using CGH.

3.3 Isolation of HRS cells by LMD

LMD DOP-PCR was performed on cytospins prepared from single cell suspensions of fresh or viably-frozen lymph node biopsies as described in the previous chapter. Identification of HRS cells was based on positive staining for CD30 antigen coupled with morphological criteria (Figure 1). Fifty single cells were laser microdissected from each case into the caps of five PCR tubes (10 cells per cap) by use of the Leica Laser Microdissection system (Leica Microsystem, Milton Keyes, UK). DOP-PCR reactions including 60 pg of normal male and female DNA (Promega, Southampton, UK) were used to generate reference control DNA for CGH. Replicates of 10 IM9 cells and 10 Daudi cells obtained by LMD and aliquots of 60 pg of MPE600 DNA were subjected to DOP-PCR for use in CGH validity testing experiments.

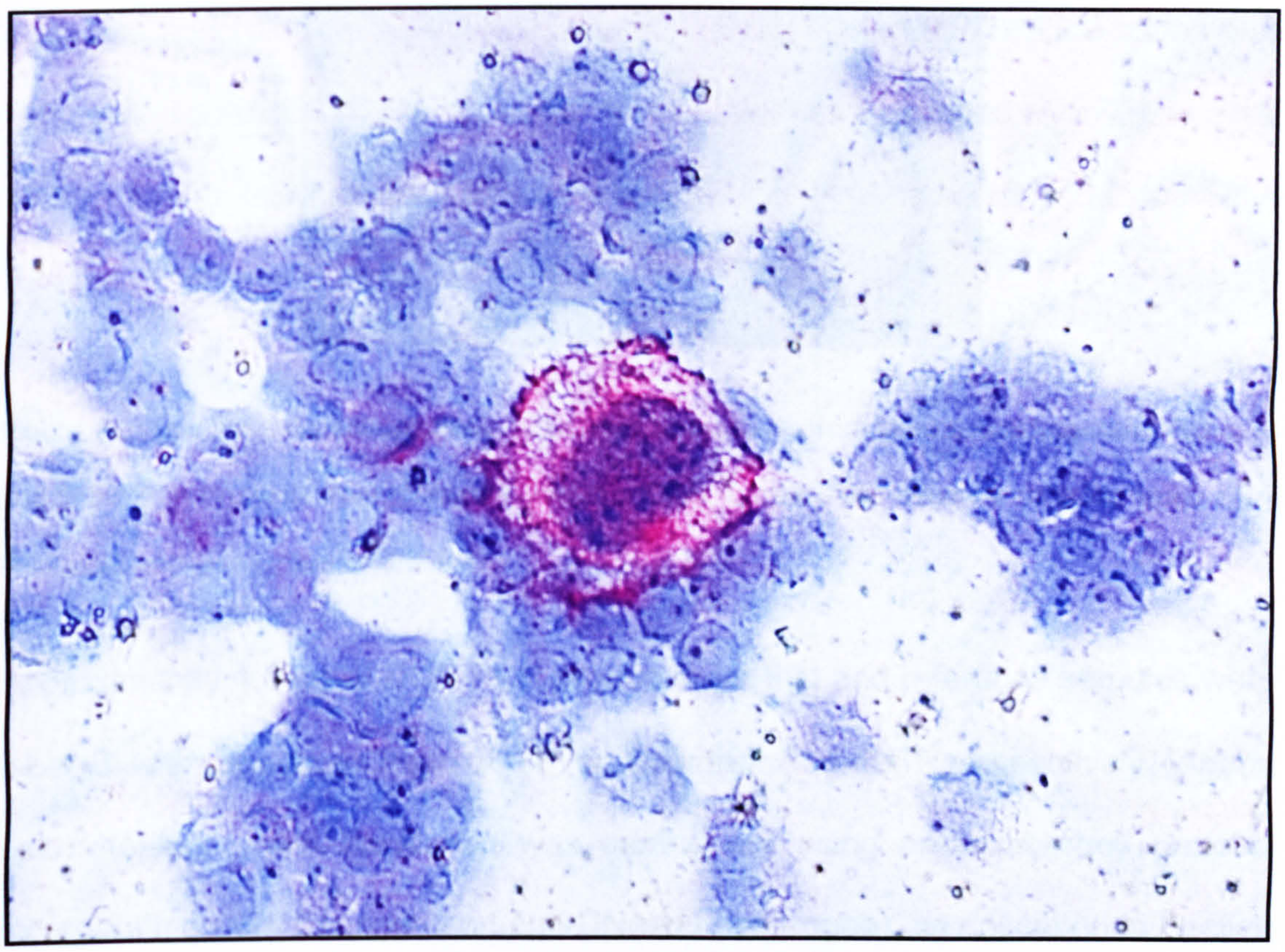

Figure 3.1 CD30-positive HRS cell on PENfoil, cytospin preparation (x 630, touludine blue counterstain).

Reaction products from the 5 DOP-PCRs from each cHL case were pooled and ethanol precipitated before labeling. The quantity of DOP-PCR products was estimated using a GeneQuant II spectrophotometer (Pharmacia Biotech) and by running one tenth of the DOP-PCR products on a 1% agarose gel (Figure 3.2).

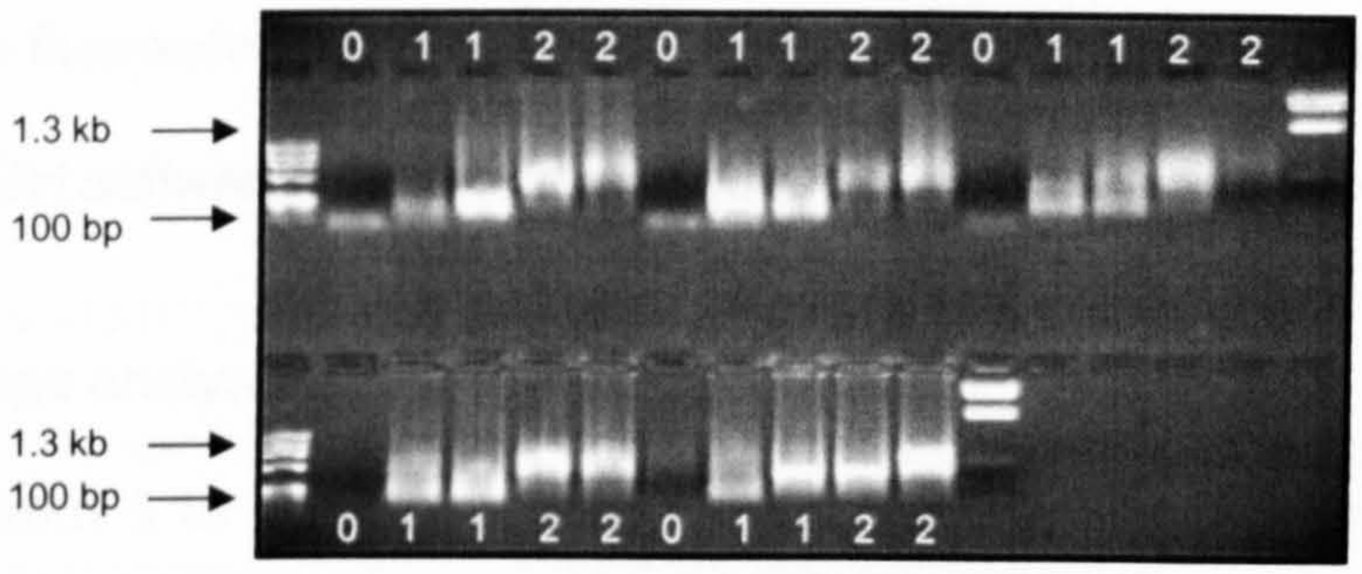

Figure 3.2 Agarose gel electrophoresis of DOP-PCR products from laser microdissected HRS cells and DOP-PCR products from reference genomic DNA. Lanes 0 are negative controls, lanes 1 are HRS cells and lanes 2 are control DNA.

Approximately 1.5 μg of DOP-PCR product from test and reference samples were labeled with SpectrumGreen-dUTP or SpectrumRed-dUTP respectively (Abbott Laboratories). Nick translation was carried out using an established protocol incorporating DNA Polymerase I and DNase I (Invitrogen), as described in Section 2.9.1.

3.4 Comparative genomic hybridisation

3.4.1 Hybridisation

Approximately 800 – 1000 ng of each labeled DNA were combined with 60 μg of human CoT-1 DNA (Invitrogen) and hybridised to normal male metaphase target slides (Abbott Laboratories) for 4 days at 37°C. After hybridisation, the slides were washed in commercially available wash buffers (Abbott Laboratories) and the chromosomes were counterstained with 4,6-diamidino-2-phenylindole (DAPI) (Abbott Laboratories). Digital images were captured using a cooled charge-coupled device camera (Photometrics, Ottobrunn, Germany) connected to a Zeiss

Axioskop fluorescence microscope (Carl Zeiss, UK). Images were analyzed with Quips CGH software (Abbott Laboratories).

3.4.2 Image analysis

Results from 5 to 10 optimal target metaphases were combined to produce the final CGH result in each case. Chromosome 19, centromere and telomeric regions were excluded from the analysis because of possible non-uniform or incomplete blocking of repeated sequences in these regions (Kallioniemi et al. 1994). Since the tumour specimens and reference DNA were not sex-matched, the X and Y chromosomes were also excluded.

3.4.3 Analysis of results

Results were analysed by visual inspection of an ideogram containing results from all cases, and by recording amplifications and deletions of both chromosome arms. Recurrent imbalances of chromosomal arms were identified as gains affecting ≥30% of cases or losses affecting ≥25% of cases. A lower threshold was applied to losses because CGH is less sensitive in the detection of deletions. The total number and presence or absence of recurrent imbalances were analysed with respect to age group, sex, histological subtype, outcome and EBV status of tumours. Age groups were defined as those <35 years old, 35-55 years old and >55 years old. Poor outcome was defined as relapse or death. Statistical testing used the two sample t-test, Fisher's exact test (two-tailed) and Kruskal-Wallis test. Analyses were implemented using SPSS (SPSS, Woking, UK).

3.5 Results

In validity testing experiments, all the expected abnormalities were identified using DOP-PCR products from the MPE600 cell line whereas normal profiles were obtained using laser microdissected IM9 and Daudi cells. Optimisation experiments using material obtained by LMD revealed that amplification of 5 replicates of 10 HRS cells gave a better yield and size range of DOP-PCR products than amplification of 50 HRS cells in a single tube (see chapter 2). Satisfactory CGH results were obtained for the 20 cHL cases analysed using this strategy and results are presented in Figure 3.3 and Table 3.2.

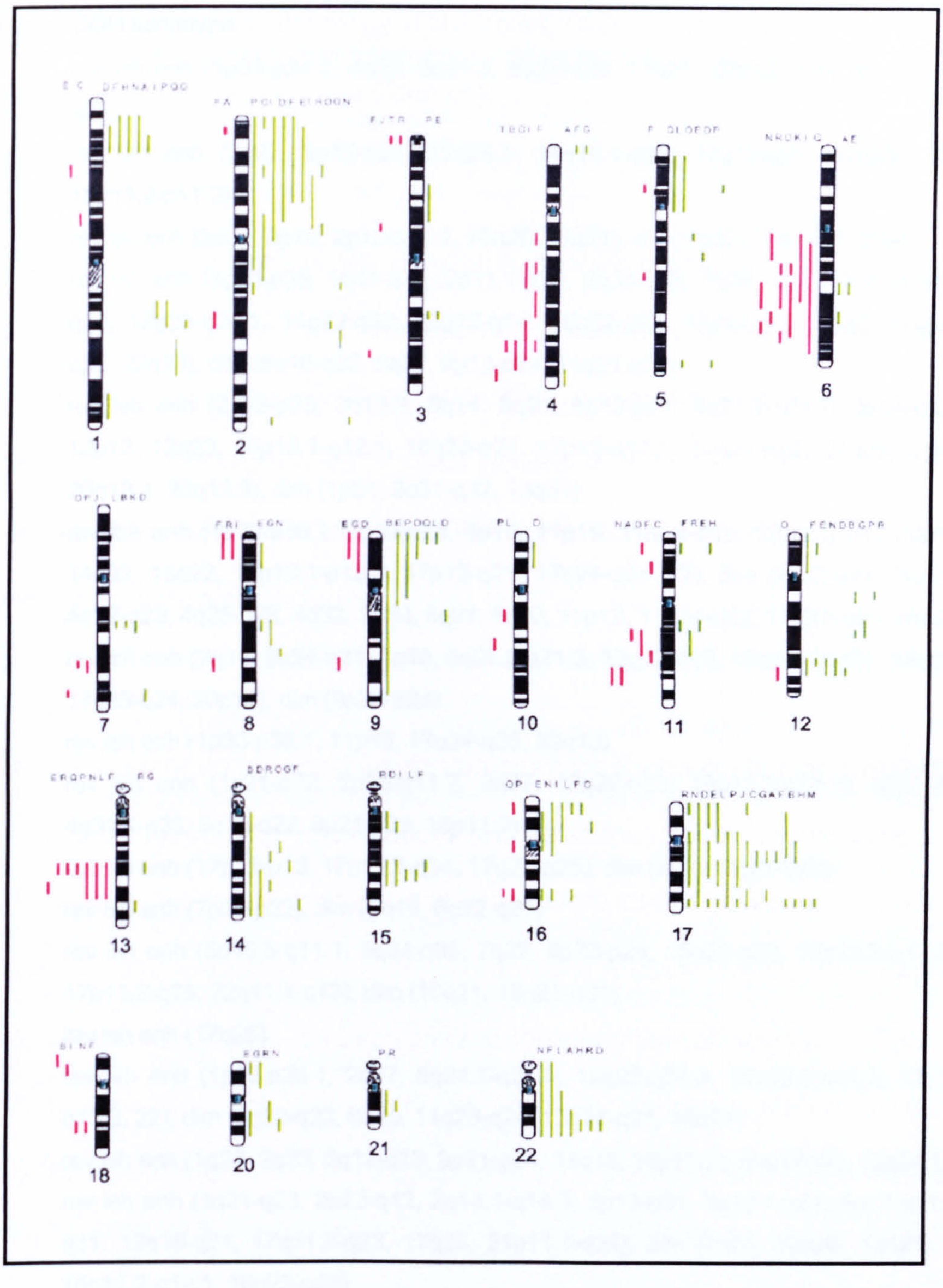

Figure 3.3 CGH results from 20 cHL. Bars to the left of the ideograms indicate regions of copy number loss and bars to the right of the ideograms indicate regions of copy number gain. Chromosome 19 was excluded because of false positives in negative controls, and the X and Y chromosomes were excluded because of non-sex-matched reference and tumour DNA.

Case	CGH karyotype
A	rev ish enh (1p33-p34.2, 4q35, 6p21.3, 6q22-q24, 17q21, 22q12-q13), dim (2p24, 11q22-q24)
B	rev ish enh (7q22, 9p13-q34, 12q24.1, 14q11.1-q32, 17q12-q21, 17q25), dim (4q32, 18p11.2-p11.3)
C	rev ish enh (2p24, 2p16, 2p12-q14.1, 10q26, 17q21), dim (1p22, 11p14, 11q14)
D	rev ish enh (1p33-p35, 1q41-q44, 2p11.1-p25, 5q34-q35, 7q36, 9q12, 10p13-p15, 11q12-q13, 12q23-q24.1, 14q22-q32, 15q12-q14, 15q22-q26, 17p12-p13, 17q11.1-q22, 17q24-q25, 22q13), dim (6q16-q22, 6q27, 9p13-p24, 11q21-q22)
E	rev ish enh (2p13-p23, 3q13.2, 5q14, 6q21, 8p12-p21, 8q21.2-q21.3, 9p24-q12, 11q13, 12p13, 12q23, 16p13.1-q12.1, 16q23-q24, 17p13-q11.1, 17q21-q22, 17q25, 20p11.2-p13, 20q13.1, 20q13.3), dim (1p31, 9q31-q32, 13q31)
F	rev ish enh (1p33-p36.1, 2p13-p25, 4p16, 11p15, 11q11-q13, 12p11.2-p13, 12q23-q24.3, 14q32, 15q22, 16p13.1-p13.3, 17p13-q21, 17q24-q25, 22), dim (2q22-q24, 3q26.1, 4q13, 4q22-q23, 4q25-q28, 4q32, 5p13, 5q21, 8q23, 11p13, 11q14-q22, 13q21-q31, 18q22)
G	rev ish enh (2q13, 2q24-q31, 4p16, 8q21.2-q21.3, 12q12-q13, 12q21, 13q21, 14q21, 17q21, 17q23-q24, 20p13), dim (9p22-p24)
H	rev ish enh (1p33-p36.1, 11p15, 17q24-q25, 22q13)
I	rev ish enh (1q21-q22, 2p25-q11.2, 2q37, 15q22-q23, 16p13.3-q11.2), dim (4q23-q27, 4q31.1-q33, 6q13-q22, 8p21-p23, 18p11.2)
J	rev ish enh (17p12-p13, 17p11.1-q21, 17q23-q25), dim (3p12, 7q31-q32)
K	rev ish enh (7p21-p22), dim (6q16, 6q22-q25)
L	rev ish enh (5p15.3-q11.1, 5q34-q35, 7q22, 9p23-p24, 15q22-q23, 16p13.2-p13.3, 16q24, 17p11.2-q25, 22q11.1-q13), dim (10q21, 13q21-q31)
M	rev ish enh (17q25)
N	rev ish enh (1p32-p36.1, 2q37, 8q24.2-q24.3, 12q23-q24.3, 16p13.3-q11.2, 17, 20q13.2-q13.3, 22), dim (6q22-q23, 6q25, 11q23-q24, 13q21-q31, 18q21)
O	rev ish enh (1q23, 2q23, 5q12-q13, 9p21-p24, 14q13, 16p11.2), dim (7q36, 12q24.1, 16q23)
P	rev ish enh (1q21-q23, 2p23-q12, 2q14.1-q14.3, 3p13-p21, 3q13.1-q21, 5p13-q11.2, 9p24-q11, 12q15-q21, 17q11.2-q23, 17q25, 21p11.1-q21), dim (7q31, 10q26, 13q21, 16p13.3, 16q11.2-q12.1, 16q23-q24)
Q	rev ish enh (1q31-q32, 5p11-p15.3, 9p23-p24) dim (4q32-q34, 6q16-q27, 13q21)
R	rev ish enh (2p16, 2p11.1-p11.2, 11q14-q21, 12q13, 13q32, 14q21, 14q23-q24, 15p11.1-q14, 15q21-q24, 17p13, 17p11.1-q23, 17q25, 20q11.2-q13.1, 21q21-q22, 22q13), dim (3p25-p26, 6q12-q15, 6q24-q25, 8p21-p23, 13q21)
S	nil
T	rev ish enh (2p14-q11.2), dim (3p26, 4q35)

Table 3.2 CGH karyotype of 20 cHL.

Gains affecting ≥30% and losses affecting ≥25% of the cases were identified. Twelve chromosomal arms were involved; only one case did not have any abnormality affecting these arms. The most frequent gains were on chromosome 17, with gains on 17q and 17p detected in 14/20 and 8/20 cases, respectively. The most commonly over-represented regions were 17q21 and 17p13. Other frequent gains involved 2p (40%), 12q (40%), 22q (35%), 9p (30%), 14q (30%), 16p (30%), with minimal overlapping regions at 2p23-13, 12q24, 22q13, 9p24-23, 14q32, 16p13.3 and 16p11.2. The most frequent losses involved 13q (35%), 6q (30%), 11q (25%) and 4q (25%), with corresponding minimal overlapping regions at 13q21, 6q22, 11q22 and 4q32. Analysis of individual chromosome arms revealed significantly more gains of 2p and 14q in the older adult cases (p-value = 0.038 and 0.022, respectively); losses of 13q were associated with a poorer outcome (p-value = 0.049). The number of chromosomal imbalances ranged from 0-21, with a mean of 10. Amplifications were more frequent than deletions with a mean value of 7 gains and 3 losses.

Results from CGH analysis of the EBV-negative, HL-derived cell lines L428, L1236 and KMH2 showed a resemblance to results from analysis of primary HRS cells (Table 3.3). Shared abnormalities included gains of 2p, 12q, 17p, 16p, 14q and losses of 13q, 6q, 4q and 11q. In contrast the EBV-positive HL-cell line, L591, showed a different CGH pattern with fewer abnormalities; these included gains in 7q and 9p and losses of 8p, 9q and 22q.

Cell line	CGH karyotype
L428	rev ish enh (2p13-p16, 2q37, 6p12-q24, 7q22-q32, 8q22-q24.3, 9p23-p24, 11q23-q25, 12p11.1-p11.2, 16q22-q24, 17p13, 17p11.1-q12, X), dim (4p16, 4p12-p13, 11q22, 13p13-q34, 14q32, 15p11.1-p11.2, 18p11.2-p11.3, 18q12-q23, 21p11.2-p13, 22p11.1-p13)
L1236	rev ish enh (1q22-q44, 2p13-p25, 3p25-p26, 3q21, 3q24, 5q23-q31, 7p21, 7q11.2-q22, 8q23-q24.3, 9p11-p24, 11q23-q25, 12p13-q11, 12q13, 15q22-q23), dim (4p14-p15.3, 4p11-p12, 4q33-q35, 6q16-q22, 8p23, 9q31-q34, 10q21-q26, 11p14, 12q21-23, 13p11.1-p13, 13q12-q21, 13q31-q33, 14p13, 14q11.2, 15p11.1-p13, 16p13.1, 17q25, 21p11.1-p13, 22p11.1-p13, 22q12-q13)
KMH2	rev ish enh (1p35-p36.3, 1q21-q44, 2p11.2-p16, 2q22-q31, 3q25-q29, 5p11-p13, 6p21.3-p25, 7q33-q36, 8p23, 9p23-p24, 9p12-p13, 10q25, 11p14-p15, 12q21-q24.3, 14q21-q32, 15q21-q24, 16p11.1-p13.2, 16q21-q24), dim (3p25-p26, 3p12-q21, 4q34-q35, 5p15.2-15.3, 6p12-q24, 7p15-p22, 7q31,8p11.1-q21.3, 9q12, 10q21, 11q13-q25, 13, 14p12-p13, 14p11.1-q13, 15p12-p13, 15p11.1, 16q11.2-q12.1, 18p11.3, 18q11.1-q23, 20p13, 20p11.2-q11.2, 21p13, 22p13, 22q13)
L591	rev ish enh (7p21-p22, 7q11.2-q22, 9p12-p24, 11q23-q25, X), dim (7q31-q36, 8p12-p23, 14q31-q32)

Table 3.3 CGH karyotype of Hodgkin lymphoma-derived cell lines.

3.6 Discussion

Analysis of 20 cases of cHL using CGH revealed recurrent chromosomal imbalances. Imbalances on 12 chromosomal arms were considered recurrent as these arms were affected in ≥30% of cases for gains and ≥25% of cases for losses (Figure 3.3). The most frequent abnormality was gain on 17q (70%). Other frequent gains involved 2p (40%), 12q (40%), 17p (40%), 22q (35%), 9p (30%), 14q (30%) and 16p (30%). The most frequent losses involved 13q (35%), 6q (30%), 11q (25%) and 4q (25%).

Two other laboratories have investigated chromosomal imbalances in cHL using CGH (Ohshima et al. 1999; Joos et al. 2000; Joos et al. 2002). Due to the difficulty

of working with cHL, different techniques were used to obtain HRS cells and the results of the studies were not consistent. In the present study HRS cells were obtained by LMD from cytospin preparations that had been immunostained using CD30 monoclonal antibodies. This method of collection of HRS cells offers advantages over previous methods used in CGH experiments in that single HRS cells were individually selected using stringent criteria, and the use of cytospins prepared from viable material ensured that complete cells were captured, hence the robust nature of this study.

Ohshima et al. (1999) sorted CD30-positive giant cells by flow cytometry using an automatic cell-deposition unit. Most of the abnormalities reported in this study were not detected in the present study or that of Joos et al. (2000). In contrast, the data presented here are largely similar to those of Joos et al. (2000, 2002). These workers initially isolated CD30-positive HRS cells by micromanipulation from cytospins and later used laser microdissection with identification of HRS cells by morphology alone. Similar abnormalities, including gains of 2p, 12q, 17p, 17q, 9p, 16p and 22q and loss of 13q are seen in the two data sets but the frequency of these abnormalities is somewhat different (Figure 3.4). The similarities add weight to the idea that cHL is associated with a consistent set of chromosomal imbalances. Variations in the proportion of older adult cases, EBV associated cases and histological subtypes might all contribute to the differences in frequency of recurrent imbalances observed between ours and Joos et al.'s data. Although not of statistical significance, higher number of chromosomal imbalances is observed in non-EBV associated cases.

It is not possible to include Oshima et al. (1999)'s data into our comparison analysis. They have included in their analysis many chromosomal regions where it is known to create false signals, such as most telomeric and centromeric regions. In particular they have included chromosome 19 and 1p in their analysis which are excluded in both ours' and Joos' study. Their CGH ideogram demonstrated in many instances both gains and losses in the same chromosomal regions. This would cast serious doubt into the homogeneity of the tumour samples obtained for CGH. The three most frequent imbalances described were losses on 16q11/21, gain on 1p13 and a gain on 7q35/36. Chromosome 1p13 is located right across the centromeric region and 7q35/36 is at the telomeric region. Losses on 16q11/21 would be the only abnormality considered to be recurrent by our method of analysis as it spanned a substantial length of the long arm of chromosome 16 away from telomeric and centromeric regions and there are no contradictory gains in the same region.

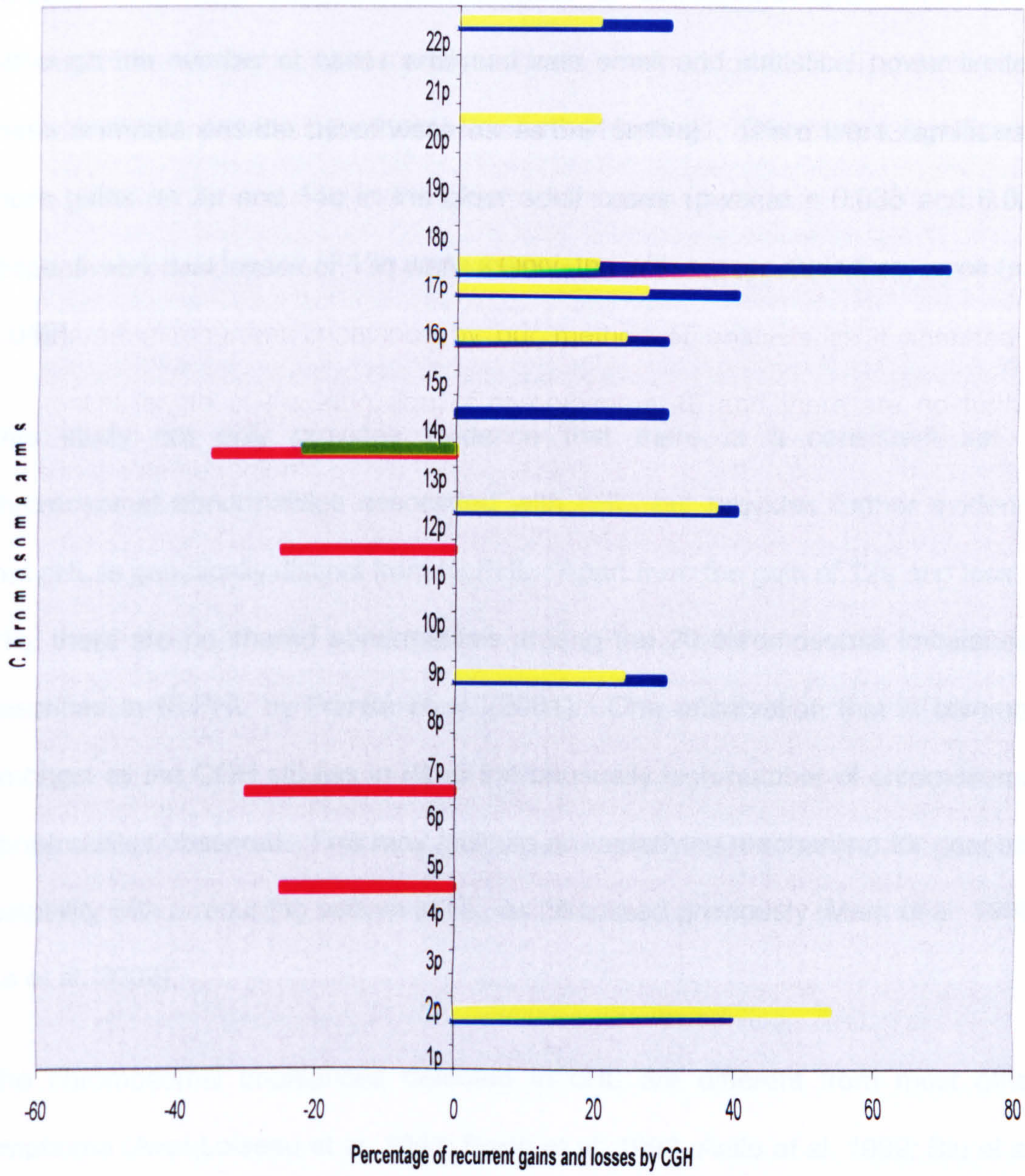

Figure 3.4. Comparison of recurrent gains and losses of CGH data from Joos et al. 2002 and Chui et al. 2003. Yellow lines (2p, 9p, 12q, 16p, 17p, 17q, 20q, 22q) are recurrent gains (>20%) by Joos et al. 2002. Blue lines (2p, 9p, 12q, 14q, 16p, 17p, 17q, and 22q) are recurrent gains (>30%) by Chui et al. 2003. Green line (13q) is recurrent losses (>20%) by Joos et al. 2002. Red lines (4q, 6q, 11q, and 13q) are recurrent losses (>25%) by Chui et al. 2003.

Results were also analysed with respect to clinicopathological parameters. Although the number of cases analysed was small and statistical power limited, these analyses provide hypotheses for further testing. There were significantly more gains on 2p and 14q in the older adult cases (p-value = 0.038 and 0.022 respectively); and losses of 13q were associated with a poor clinical outcome (p = 0.049).

This study not only provides evidence that there is a consistent set of chromosomal abnormalities associated with cHL, but provides further evidence that cHL is genetically distinct from NLPHL. Apart from the gain of 12q and loss of 11q, there are no shared abnormalities among the 20 chromosomal imbalances described in NLPHL by Franke et al. (2001). One observation that is common amongst all the CGH studies in HL is the unusually high number of chromosomal abnormalities observed. This may indicate an underlying mechanism for genomic instability with a recurring pattern in HL, as discussed previously (Mark et al. 1998; Re et al. 2002).

The chromosomal imbalances detected in cHL are different from most other lymphoma (Avet-Loiseau et al. 1997; Barth et al. 1998; Aalto et al. 1999; Siu et al. 1999; Barth et al. 2001; Tsukasaki et al. 2001; Allen et al. 2002), with the exception of primary mediastinal B-cell lymphoma (PMBCL). PMBCL is the only NHL which shares frequent gains of 2p and 9p and losses of 13q, 6q and 4q (Bentz et al. 2001; Palanisamy et al. 2002). This is of particular interest since both

tumours have a tendency to occur in young females, have a predilection for the mediastinum, and lack expression of surface Ig.

Gain of chromosome 17q was the most frequent abnormality found in this CGH study of cHL. Alteration of 17q was also one of the more frequently found abnormalities in previous cytogenetic studies of cHL (Sarris et al. 1999). Chromosomal gains in cHL, including gains of 2p, 9p and 12q, have been further investigated by FICTION (Fluorescence-immunophenotyping and Interphase Cytogenetics as a Tool for Investigation Of Neoplasm) (Weber-Matthiesen et al. 1992) and gene amplification of REL (2p15-p16), JAK2 (9p24) and MDM2 (12q24) in cHL have been confirmed (Joos et al. 2000; Kupper et al. 2001; Joos et al. 2002). Involvement of possible oncogenes on chromosome 17q has not been studied (Stokke et al. 2001). However, many genes that have been previously implicated in cHL, including genes involved in, or regulated by, NF-κB activation, and genes encoding chemokines and STATs reside in 17q.

Several possible mechanisms of NF-κB activation have been described in cHL including expression of the EBV LMP1 protein and mutation of *IkBa* (Cabannes et al. 1999; Lee et al. 2001). Overexpression of the serine / theronine protein kinase NIK (17q21) has also been shown to cause NF-κB activation in human embryonic kidney cells, and it is therefore possible that this mechanism also plays some role in cHL (Malinin et al. 1997). NF-κB activation leads to up-regulation of a number of genes thought to contribute to the pathogenesis of cHL, including those encoding chemokines and STATs. Aberrant activation of JAK-STAT signaling has

been described in human cancers and many tumours contain increased levels of activated nuclear STATs, frequently STAT3 or STAT5 (Watson and Miller 1995; Weber-Nordt et al. 1996; Bowman et al. 2000). Activated STAT3 (17q21) was shown to be highly expressed in the HRS cells of cHL by ICC (Chen et al. 2001; Garcia et al. 2002) and STAT5a (17q11.2), a transcription factor linked to cell growth control, was found to be constitutively active in the HRS cells in all 24 cases of cHL studied, although only weakly expressed in NLPHL (Hinz et al. 2002).

Eosinophils are conspicuous in some cases of cHL and eotaxin, encoded by a chemokine gene on 17q21, is a potent inducer of eosinophil chemotaxis and angiogenesis (Ponath et al. 1996; Salcedo et al. 2001). In 1999, (Teruya-Feldstein et al. 1999)described a high level of eotaxin expression in HRS cell, however (Jundt et al. 1999) have suggested that the eotaxin is produced by surrounding fibroblasts following induction by TNF-α released by HRS cells. Many other chemokines including I309 (17q11.2), MCP-4 (17q11.2) and RANTES (17q12) are highly expressed in HL and thought to play a role in recruitment of reactive cells to these tumours (Maggio et al. 2002).

Survivin (17q25) is overexpressed in most cancers of the lung, colon, pancreas, prostate and breast (Ambrosini et al. 1997). Survivin has previously been found to be highly expressed in HL by RT-PCR (Shinozawa et al. 2000) and expression of survivin protein has recently been confirmed by Garcia et al. (2002). In the latter study, survivin was detected in the HRS cells of over 89% of cHL cases.

Overexpression of survivin can delay apoptosis by directly inhibiting effector caspases or by protecting the microtubules of the mitotic-spindles and related structures from caspase cleavage (Reed and Bischoff 2000).

Gain of 17p was found in 40% of patients in this study. Overexpression of TP53 (17p13.1) has been found in a high percentage of cases of HL but there is no correlation between overexpression of TP53 and the presence of gene mutation in cHL (Chen et al. 1996; Elenitoba-Johnson et al. 1996; Sanchez-Beato et al. 1996b; Montesinos-Rongen et al. 1999).

Loss of 13q and 6q is commonly seen in other haematological malignancies, and thus could relate to a common haemopoietic origin (Avet-Loiseau et al. 1997; Barth et al. 1998; Siu et al. 1999; Bentz et al. 2000; Nagy et al. 2000; Barth et al. 2001; Stokke et al. 2001; Tsukasaki et al. 2001; Allen et al. 2002; Palanisamy et al. 2002). Although the retinoblastoma (RB1) gene is located on 13q14, this tumour suppressor gene does not appear to be the target of deletions in cHL (Weiss 1995; Guenova et al. 1999; Kanavaros et al. 2000) and other lymphoma (Liu et al. 1995; Stilgenbauer et al. 1998). Expression of the RB protein has been found in most cases, suggesting the presence of another tumour suppressor gene on 13q13-q21. Loss of 6q has been associated with a poor prognosis in DLBCL (Harada et al. 2001), follicular lymphoma (Tilly et al. 1994) and acute lymphoblastic leukaemia (ALL) (Merup et al. 1998) but as yet no tumour suppressor gene has been identified in the 6q21-q27 region (Offit et al. 1993; Hauptschein et al. 1998).

With the exception of L1236, HL-derived cell lines have an uncertain relationship to the tumours from which they were derived (Wolf et al. 1996). Results of CGH analysis of the EBV-negative HL-derived cell lines showed a marked resemblance to the results from primary HRS cells, including gains of 2p, 12q, 15q, 17p, 16p, 14q and losses of 13q, 6q, 4q and 11q. In contrast, the EBV-positive derived cell line L591 showed a very different CGH pattern with few abnormalities. This observation in L591 may relate to the EBV status of the cells or suggest a non-HRS cell origin (Drexler 1993).

In summary, this CGH study has identified a set of recurrent chromosomal abnormalities associated with cHL with gain of 17q being the most frequent abnormality in this series of cHL. Abnormalities of 17q are infrequent in NHL or NLPHL; this finding, coupled with current knowledge of gene expression in cHL, suggests that genes present on 17q may play an important role in the pathogenesis of cHL.

Chapter 4

STUDY OF REL AND STAT3/5A GAIN IN HODGKIN AND REED-STERNBERG CELLS AND CORRELATION WITH I KAPPA B ALPHA MUTATION STATUS

4.1 Introduction

In Hodgkin lymphoma (HL), chromosomally aberrant clones are only detected in 20-30% of cases. Karyotypes typically are hyperploid, often in the triploid or tetraploid range, and show very complex chromosome aberrations (Deerberg-Wittram et al. 1996). In the majority of analysed cases, only normal metaphases are found. With the application of comparative genomic hybridisation (CGH), this problem has been circumvented and a set of recurrent imbalances has now been identified in classical Hodgkin lymphoma (cHL) (Ohshima et al. 1999; Joos et al. 2000; Chui et al. 2003). Due to the limitation of resolution of conventional CGH, it is not possible to pinpoint the target genes that are either amplified or deleted. The traditional method to look for gene amplification is by FISH on metaphase spreads from the tumour cells using a specific gene probe, but this is not possible for HL as the percentage of Hodgkin and Reed-Sternberg (HRS) cells with metaphases is far below the detection limit of conventional FISH (Poddighe et al. 1991).

Previously, combining fluorescence immunophenotyping and *in situ* hybridisation was limited to studying numerical aberrations using centromeric probes (CEP) or genetic aberrations with a very limited number of gene specific probes. With the advent of the Human Genome Project (HGP), a full draft sequence of the human genome has been published (Lander et al. 2001). This has greatly facilitated the discovery and understanding of many new genes. FISH mapped clones covering the entire chromosomes and encoding many known and unknown genes are now in the public domain. By extracting the DNA from bacterial artificial chromosome

(BAC) clones and labelling them with fluorochromes, it is possible to prepare a large number of gene specific FISH probes (McPherson et al. 2001). When combined with ICC this technique is termed fluorescence immunophenotyping and interphase cytogenetics as a tool for investigation of neoplasms (FICTION) (Weber-Matthiesen et al. 1992). With this method, cells can be characterised by their immunophenotype and analysed with regard to gene amplification. In this way, cells with tumour-associated immunophenotype can be evaluated selectively. In cHL, the tumour cells strongly express the CD30 antigen and have a characteristic morphology, while the surrounding lymphocytes are mostly CD30 negative. This advantage of the FICTION method allows accurate interphase cytogenetic studies in cHL, even if the extremely low number of tumour cells limits the application of FISH. This chapter will demonstrate how FICTION has been used to confirm previous CGH findings and to provide further information on the genes without the need for tumour metaphase spreads.

4.1.1 I kappa B alpha

The inhibitor of kappa B (IκB) family consists of several members including IκBα, IκBβ, IκBε, and BCL3 (see Section 1.11.1). They are characterised by their 6-7 ankyrin repeats, which allow them to interact with members of the REL family of transcription factors. The human *IkBa* gene has six exons that span approximately 3.5 kb (Ito et al. 1995). IκB proteins are not only responsible for cytoplasmic sequestration of NF-κB in resting cells, but they also associate with NF-κB in the nucleus, where they inhibit NF-κB DNA binding and promote transport of NF-κB to

the cytoplasm, thus terminating transcription and resetting the switch (Simeonidis et al. 1999).

4.1.2 REL

The *c-rel* gene encodes for a member of the NF-κB family of transcription factors, and is located on chromosome 2p15. The high frequency of chromosomal gains on 2p in a region including the *c-rel* locus suggests an alternative mechanism leading to NF-κB activation in cHL, namely a gene dose effect resulting in an increased expression of REL (Joos et al. 2002). Detailed analysis using FICTION on individual HRS cells previously demonstrated that REL gains correlated with the presence of nuclear c-Rel by ICC (Barth et al. 2003).

4.1.3 STAT3 and STAT5a

Aberrant activation of the JAK-STAT signaling pathway has been described in human cancers and many tumours contain increased levels of activated nuclear STATs, frequently STAT3 or STAT5 (Watson and Miller 1995; Weber-Nordt et al. 1996; Bowman et al. 2000). Activated STAT3 (17q21) was shown to be highly expressed in the HRS cells in cHL by ICC (Chen et al. 2001; Garcia et al. 2002) and STAT5a (17q11.2), a transcription factor linked to cell growth control, was found to be constitutively active in the HRS cells in all 24 cases of cHL studied, although only weakly expressed in NLPHL (Hinz et al. 2002).

The work described in this chapter aimed to determine whether *c-rel* amplification and *IkBa* mutation are mutually exclusive events in the pathogenesis of cHL. In

addition, analysis of STAT3/5a genes by FICTION was performed in an attempt to identify the critical genes involved in 17q gains.

4.2 Materials and methods

4.2.1 Clinical cases and experimental design

4.2.1.1 FICTION cases

Ten cHL cases with sufficient tumour material were selected from our database. Six of the ten were used in the CGH study described in the last chapter. Cytospins were prepared as described in Chapter 2. Imprints and paraffin sections of the 10 cHL cases were also obtained. Slides were taken to the Institute of Human Genetics, Kiel, Germany in a slide box at room temperature. The REL and STAT3/5a probes used were previously developed and validated by Dr. J. I. Martin-Subero and Jennifer Reimke (see Table 4.1) (Institute of Human Genetics, Kiel, Germany). The newly precipitated FISH probes were tested on normal metaphase slides prior to being used on clinical samples. The experiments were carried out twice and data collected blindly by 2 independent observers without prior knowledge of CGH results. Detailed description of methodology can be found in Section 2.10.

4.2.1.2 *IkBa* mutation in HRS cells

Twenty HRS cells from each of the 10 cases were laser microdissected and lysed as described in Section 2.6.2. The resultant DNA samples were subjected to a semi-nested PCR amplifying exons 1 to 6 of the *IkBa* gene section. The PCR products were then purified, and directly sequenced using BigDye Terminator v3.1

Cycle Sequencing Kits and an ABI Prism 3100 Genetic Analyzer (Applied Biosystems) as described in Chapter 2. The sequencing part of this project was performed by Ms. Annette Lake. The data obtained from mutational analysis were correlated with FICTION results and EBV status from these 10 cases.

4.2.2 FISH probes

4.2.2.1 Self-designed probes

The BAC clones (see Table 4.1) used in development of FISH probes applied in this chapter were derived from the BAC library of the Roswell Park Cancer Institute (RPCI-11) (http://bacpac.med.buffalo.edu). Clones were purchased from the German Resource Centre for Genome Research (http://rzpd.de) or kindly provided by the Sanger Centre (http://www.sanger.ac.uk).

Clone reference	Description	Fluorescence label
498O5	PAPLOG (2p16.1)	SpectrumGreen
373L24	REL (2p16.1)	SpectrumGreen
156E6	Upstream of STAT3/5a at 40.1 MB (17q21.2)	SpectrumOrange
400F19	Downstream of STAT3/5a at 40.6 MB (17q21.2)	SpectrumGreen

Table 4.1 List of all BAC clones used as self-designed FISH probes in this study.

4.2.2.2 Commercial probes

In addition to self-designed probes, commercial CEPs were used in this study (Table 4.2).

Probe	Description	Source
CEP2	Specific for the centromere of chromosome 2 (locus D2Z1)	Abbott Laboratories
CEP17	Specific for the centromere of chromosome 17 (locus D1721)	Abbott Laboratories

Table 4.2 List of centromeric probes used for FICTION in this study.

4.2.3 Antibodies

A cascade of monoclonal antibodies were used to detect the CD30 antigen present in the HRS cells of cHL (Table 4.3).

MoAb	Description	Company
CD30 (BerH2)	Mouse monoclonal antibody against human CD30 antigen	DAKO (Hamburg, Germany)
Alexa 594	Texas red-conjugated rabbit anti-mouse antibody	Molecular Probes (Invitrogen, Karlsruhe, Germany)

Table 4.3 List of all antibodies used for FICTION in this study.

4.2.4 Signal evaluation

In this study, the hybridisation signals in the HRS cells for the gene studied and its corresponding centromere were counted in a minimum of 10 CD30-positive cells and 20 negative bystander cells. This was done by 2 independent observers for each sample, without prior knowledge of CGH results. A ratio was expressed by dividing the number of gene signals by the number of centromeric signals. Gain was defined by a ratio of ≥1.5 or a median of >4 signals when centromeric signals

could not be evaluated. This criterion was applied as many HRS cells are likely to be triploid or tetraploid (Weber-Matthiesen et al. 1995a).

4.3 Results

4.3.1 Validation of REL and STAT3/5a probes on normal metaphase slides

Pooled REL/PAPLOG probe and STAT3/5a probe were hybridised onto normal metaphase slides prior to use on clinical samples. The probes were strong in signal intensity with a low background and they co-localised at the correct position on the corresponding chromosome (Figure 4.1 and 4.2).

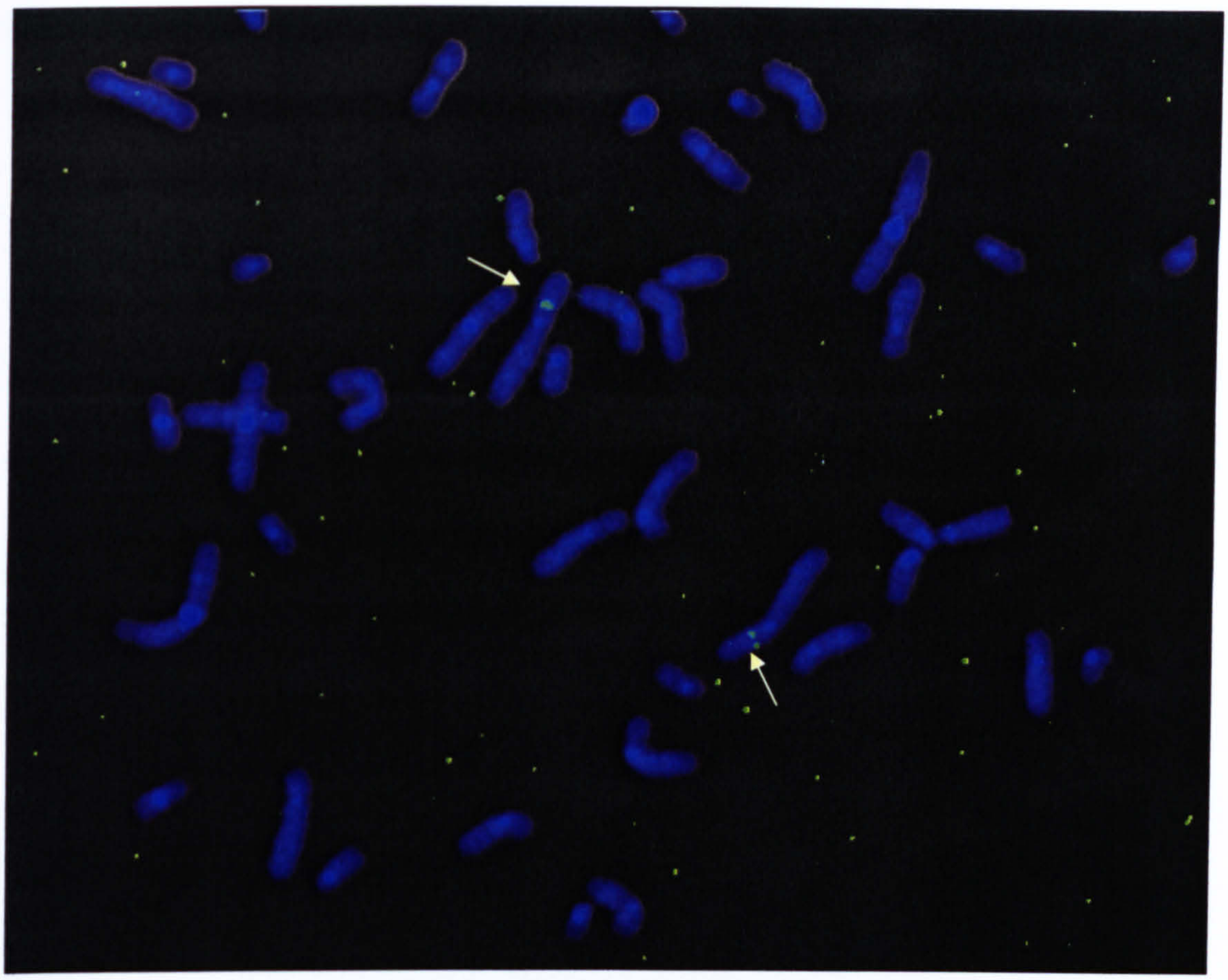

Figure 4.1 REL/PAPLOG probe hybridised to chromosome 2p16 on a normal metaphase spread slide. The REL/PAPLOG probe is labelled with SpectrumGreen and the chromosomes are counterstained with DAPI.

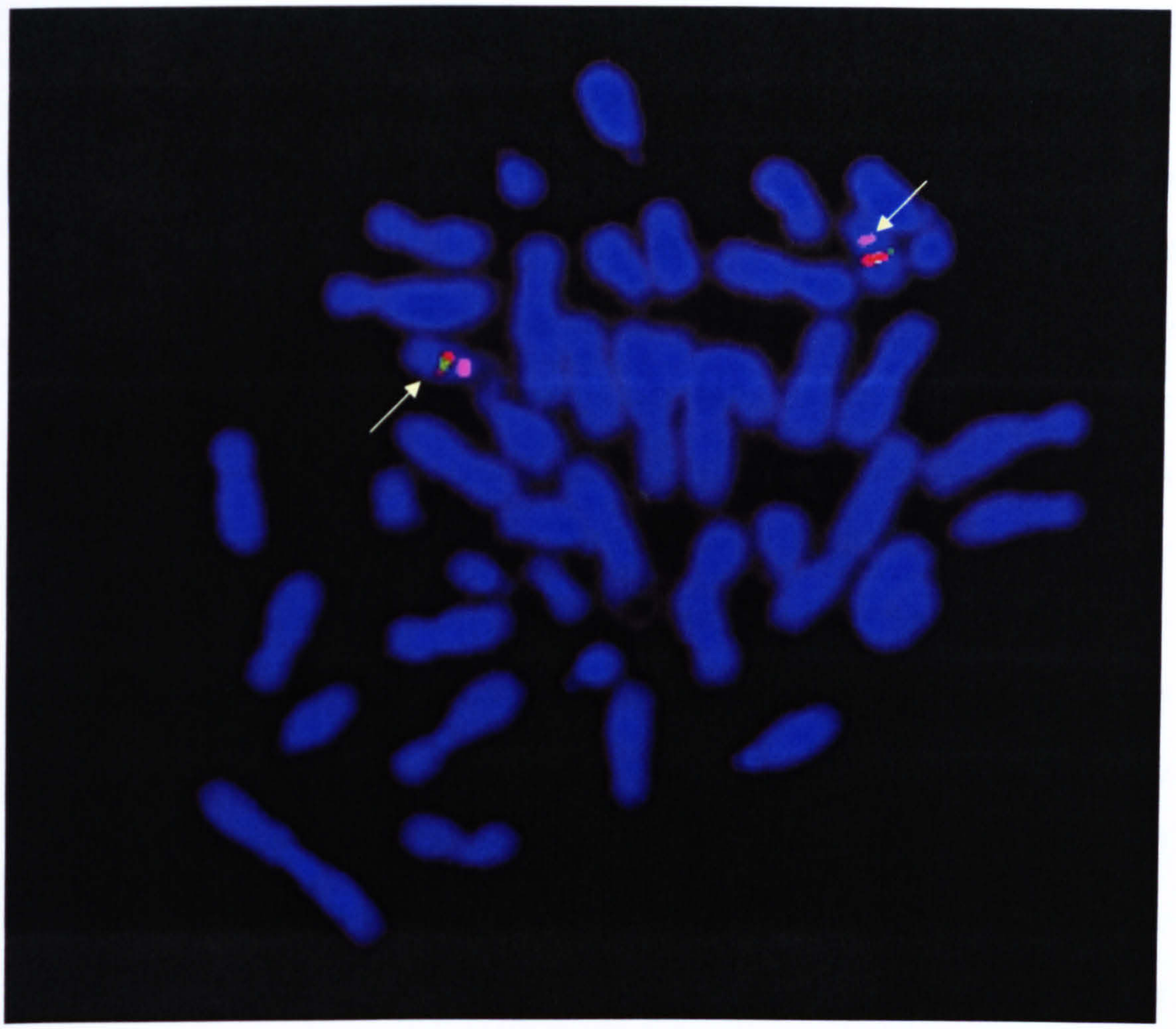

Figure 4.2 STAT3/5a probe hybridised to chromosome 17q21 on a normal metaphase spread slide. The STAT3/5a probe is labelled with SpectrumRed and SpectrumGreen; the centromeric probe for chromosome 17 is labelled with SpectrumBlue but is depicted in pink pseudo-colour).

4.3.2 Evaluation of FICTION signals for REL in HRS cells from cHL cytospins

By FICTION with application of REL and PAPLOG probes, the median numbers of REL/PAPLOG copies in the CD30-positive HRS cells ranged from 2 - 6.5 and 2 - 6 in the 10 cases of cHL evaluated by the first and second observer, respectively. Inter-observer variability was minimal (Tables 4.4a, 4.4b, 4.5a and 4.5b) and discrepancies were resolved by a third inspection with image capturing. Overall, 4

out of 10 cHL cases had a gain of REL/PAPLOG, which is in line with our CGH analyses detecting 2p gains in 8 of 20 cHL cases (see Figure 4.3). In order to relate the number of REL to the number of centromere 2 copies, FICTION was performed combining D2Z1 with the REL-BAC pool probe (see Table 4.4a and 4.4b). In 6 of the 10 cases (60%) that could be analysed, the median centromere 2 copy number exceeded the diploid range in 3 cases. In 6 of 10 cHL cases where CGH analyses were available, with the exception of 6689, good concordance was observed between 2p status by CGH and REL status by FICTION. Based on the fact that fixed thresholds in CGH do not take into account of the variation of ratio values between different experiments, different metaphases of the same slide and even within different chromosomal region, a re-examination of the red/green ratio profile was done on 6689. This revealed two distinct peaks in the 2p14 and 2p16 regions which were just below the threshold due to slightly more noise. The 2p status by CGH in 6689 was therefore interpreted as a near gain. Overall, the observations provided good supportive evidence that REL is involved in the 2p gains observed in cHL by CGH analyses. As the number of cases in this study was small, correlation with patient demographic details was not performed.

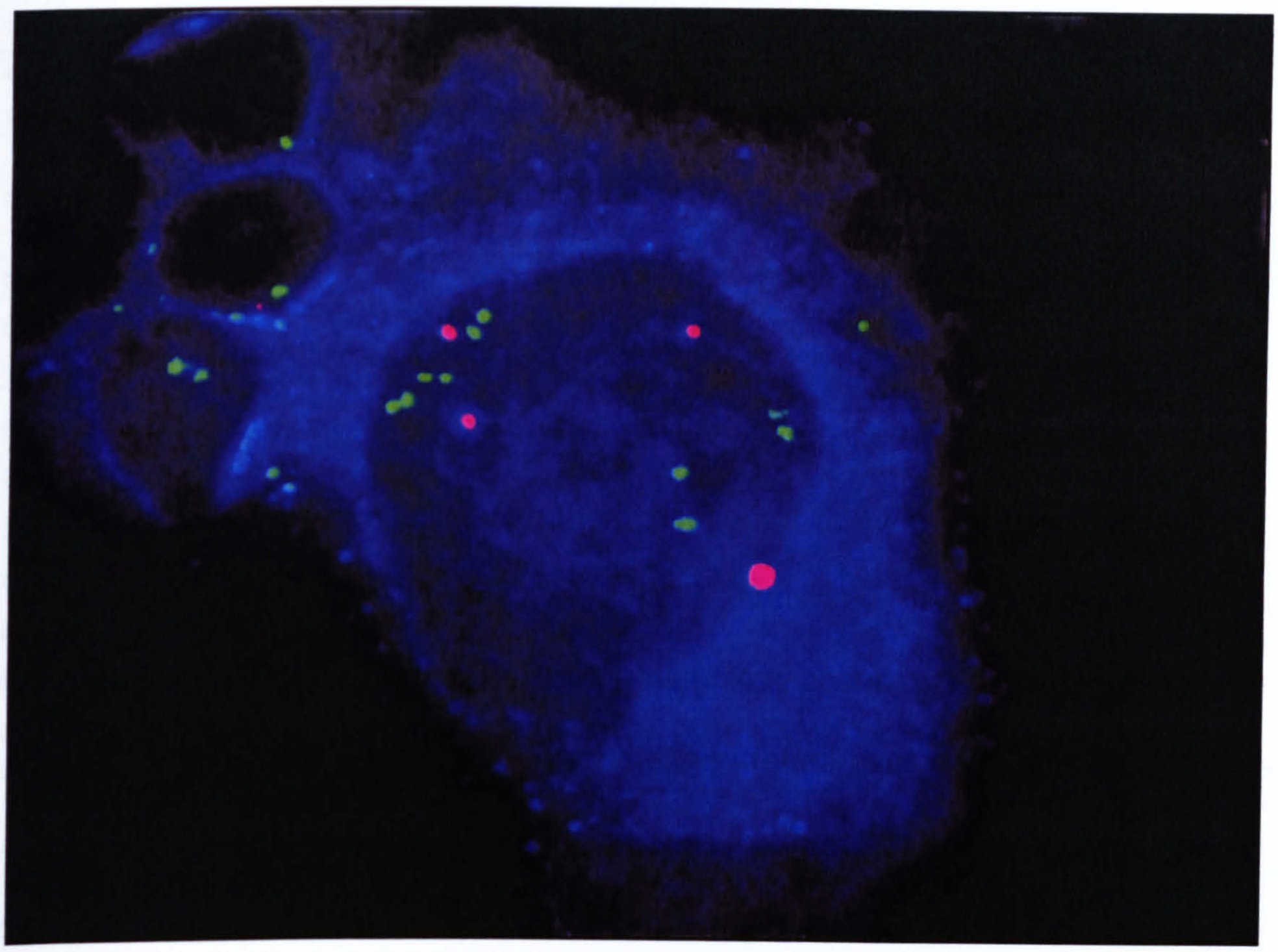

Figure 4.3 REL gain in a HRS cell. There are 10 REL signals and 4 chromosome 2 centromere signals. REL is labelled with SpectrumGreen, chromosome 2 centromere is labelled with SpectrumBlue but is pink in pseudo-colour and the cells are counterstained with DAPI.

By looking at the range of the median numbers of REL and chromosome 2 centromeres, the HRS cells in at least 7 of 10 cases appeared to have some numerical chromosomal aberrations. This is in line with previous observations by other workers (Weber-Matthiesen et al. 1995b).

Case No.	Age	Sex	HRS cells evaluated	Copies of D2Z1 median (range)	Copies of REL median (range)	REL/D2Z1	REL status	2p status by CGH
6689(L)	21	M	10	4 (2-5)	6.5 (5-9)	1.8 (1.3-4.5)	G	NG
5708(C)	46	F	16	NE	5 (4-7)	NE	G	G
5706(J)	16	F	19	2 (2-3)	2 (2-4)	1.3 (0.7-1.5)	B	B
6838	52	F	19	3 (2-3)	3 (3-4)	1.2 (1-1.5)	B	NE
6258(A)	39	M	19	2	2 (2-3)	1 (1-1.5)	B	B
5780(H)	33	M	20	NE	2 (2-5)	NE	B	B
6763	36	F	16	2 (1-2)	2 (2-3)	1 (1-2)	B	NE
6214(D)	67	M	20	NE	4 (3-8)	NE	B	G
6861	46	F	16	3.5(2-4)	4 (2-7)	1.4 (1-2.3)	B	NE
6766	51	M	19	NE	2 (2-7)	NE	B	NE

Table 4.4a REL and chromosome 2 centromere (D2Z1) signals in the HRS cells from 10 cases of cHL observed by the author (B indicates balanced, G, gain, NG, near gain and NE, not evaluable). Alphabets next to case numbers denote their corresponding CGH case identity in Chapter 3.

Case No.	Age	Sex	HRS cells evaluated	Copies of D2Z1 median (range)	Copies of REL median (range)	REL/D2Z1	REL status	2p status by CGH
6689(L)	21	M	13	4 (2-4)	5 (3-7)	1.7 (1.3-2)	G	NG
5708(C)	46	F	8	NE	5 (5-8)	NE	G	G
5706(J)	16	F	5	NE	3	NE	B	B
6838	52	F	9	NE	3 (2-3)	NE	B	NE
6258(A)	39	M	9	NE	2	NE	B	B
5780(H)	33	M	7	NE	2	NE	B	B
6763	36	F	7	2 (2-4)	2 (2-4)	1	B	NE
6214(D)	67	M	11	NE	6 (2-11)	NE	G (reconfirmed)	G
6861	46	F	6	NE	5 (4-6)	NE	G (reconfirmed)	NE
6766	51	M	NA	NE	NE	NE	NE	NE

Table 4.4b REL and chromosome 2 centromere (D2Z1) signals in the HRS cells from 10 cases of cHL observed by Dr. J. I. Martin-Subero (B indicates balanced, G, gain, NG, near gain and NE, not evaluable). Alphabets next to case numbers denote their corresponding CGH case identity in Chapter 3.

4.3.3 Evaluation of FICTION signals for STAT3/5a in HRS cells from cHL cytospins

The median number of STAT3/5a copies in the CD30-positive HRS cells ranged from 2-5 for both observers (see Table 4.5a and 4.5b). Only one case had a median STAT3/5a copy number of 5 signals but it also had a median centromere 17 copy number of 4, thus the ratio did not exceed 1.5. None of the 10 cHL cases appeared to have a gain of STAT3/5a despite 4 of 10 cases having a median centromere 17 copy number which exceeded the diploid range (see Figure 4.4). Thus, the frequent gain in 17q in cHL by CGH analyses is not associated with

genetic gain in STAT3/5a. By applying a dual colour probe flanking the STAT3/5a gene, it was possible to evaluate more than simply signal amplification. The separation of the two colour signals would indicate a break or translocation. This was indeed uncommon in all the HRS cells observed for STAT3/5a.

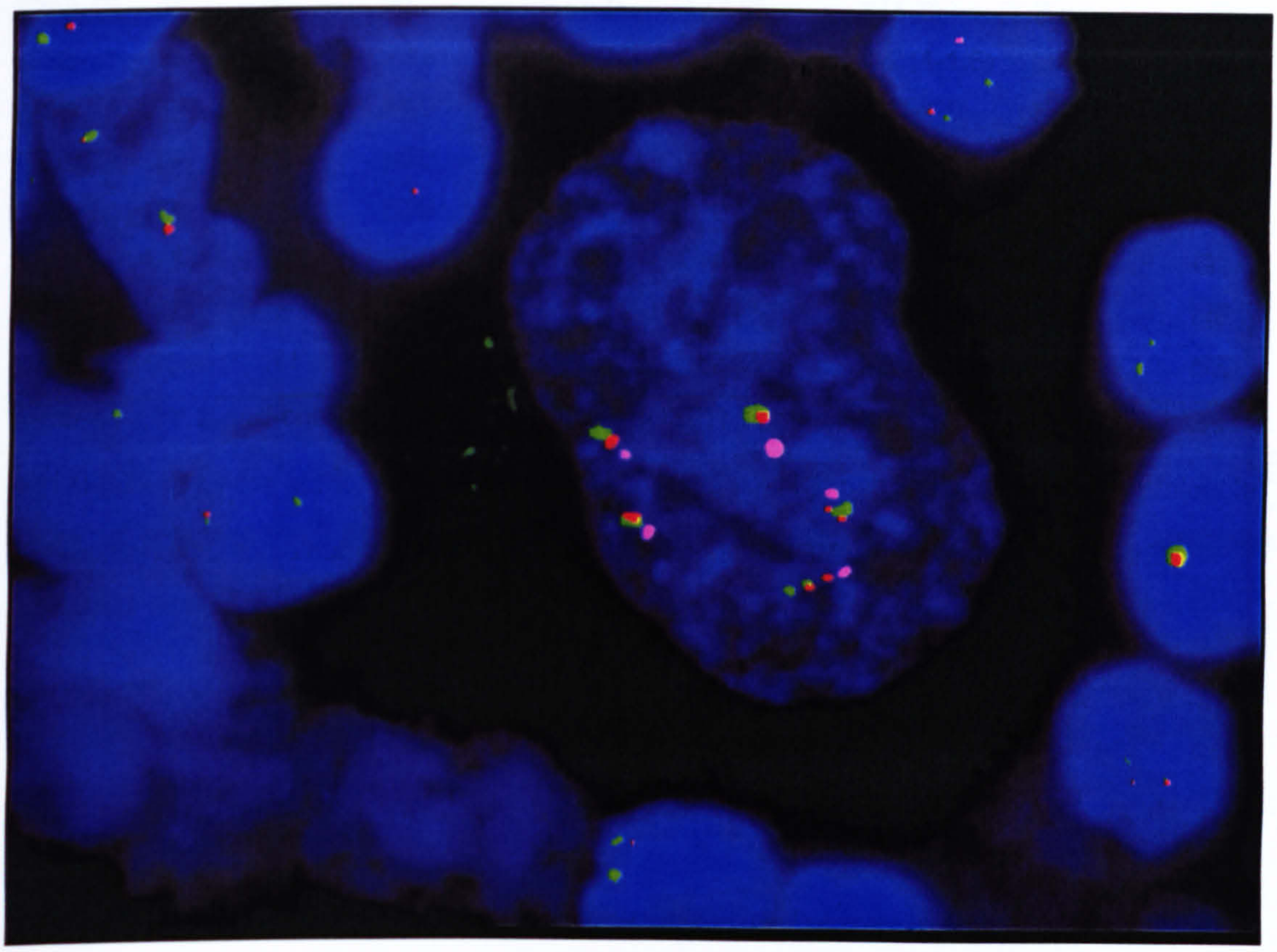

Figure 4.4 Absence of STAT3/5a gain in a HRS cell. There are 5 pairs of STAT3/5a signals and 5 chromosome 17 centromere signals (STAT3/5a is labelled with SpectrumGreen and SpectrumRed, chromosome 17 centromere is labelled with SpectrumBlue but is pink in pseudo-colour and the cells are counterstained with DAPI).

Case No.	Age	Sex	HRS cells evaluated	Copies of D1721 median (range)	Copies of STAT3/5a	STAT3/5a /D1721	STAT3/5a status	17q status by CGH
6689(L)	21	M	29	4 (4-6)	5 (4-7)	1.2 (1-1.7)	B	G
5708(C)	46	F	20	3 (2-4)	4 (2-6)	1 (1-2)	B	G
5706(J)	16	F	20	3 (2-8)	3 (2-10)	1 (0.7-1.8)	B	G
6838	52	F	14	2 (2-3)	2 (2-4)	1 (1-1.3)	B	NA
6258(A)	39	M	16	2 (1-3)	2 (2-3)	1 (0.7-1.5)	B	G
5780(H)	33	M	20	2 (1-2)	2	1 (1-2)	B	G
6763	36	F	17	2 (2-4)	2 (2-4)	1 (1-1.5)	B	NA
6214(D)	67	M	20	2 (1-2)	2	1 (1-2)	B	G
6861	46	F	18	4 (2-7)	4 (3-7)	1.3 (1-1.7)	B	NA
6766	51	M	16	2	2	1	B	NA

Table 4.5a STAT3/5a and chromosome 17 centromere (D1721) signals in the HRS cells from 10 cases of cHL observed by the author (B indicates balanced, G, gain and NA, not available). Alphabets next to case numbers denote their corresponding CGH case identity in Chapter 3.

Case No.	Age	Sex	HRS cells evaluated	Copies of D1721 median (range)	Copies of STAT3/5a	STAT3/5a /D1721	STAT3/5a status	17q status by CGH
6689(L)	21	M	12	5 (4-5)	5 (4-5)	1 (1-1.3)	B	G
5708(C)	46	F	7	3 (2-7)	4 (2-8)	1 (1-1.7)	B	G
5706(J)	16	F	10	3 (3-6)	3 (3-6)	1	B	G
6838	52	F	8	2	2	1	B	NA
6258(A)	39	M	9	2	2	1	B	G
5780(H)	33	M	10	NA	2	NA	B	G
6763	36	F	15	2 (2-6)	2 (2-8)	1 (0.7-1.8)	B	NA
6214(D)	67	M	11	NA	3 (2-4)	NA	B	G
6861	46	F	10	4 (3-4)	4 (3-5)	1 (1-1.3)	B	NA
6766	51	M	5	NA	2	NA	B	NA

Table 4.5b STAT3/5a and chromosome 17 centromere (D1721) signals in the HRS cells from 10 cases of cHL observed by Dr. J. I. Martin-Subero (B indicates balanced, G, gain and NA, not available). Alphabets next to case numbers denote their corresponding CGH case identity in Chapter 3.

Mutation of the *IkBa* gene in HRS cells occurred in 6 out of 10 cases; mutations in 3 cases were considered functionally significant. Two out of 10 cases had a partial LOH; one of these cases also had a significant *IkBa* gene mutation and the other a REL amplification (see Table 4.6). Among the 4 cHL cases with REL amplification, 3 had *IkBa* mutation although mutations in only one of these cases were considered significant. Four out of nine cases were EBV-positive but a correlation with *IkBa* gene mutations was not apparent. Overall these data show that significant mutation or LOH of *IkBa*, REL amplification and the presence of EBV in HRS cells are not mutually exclusive of each other.

Case No.	Gene status	LOH	Significance	REL gain	EBV status
6689	Exon 3 (1509 C>T) Stop codon Exon 5 (2188 Ins 7 bp)	No	Bi-allelic inactivating mutations	Yes	Negative
5708	Wild type	Partial LOH		Yes	Negative
5706	Exon 3 (1634 G>A) Splice donor	N/A	Not functionally important	No	Negative
6838	Intron 2 (1402 C>A) Intron 5 (2694 T>A) 3' UTR (3022 T>C)	Partial LOH	Potential dysregulation	No	Not known
6258	Exon 5 (2253 G>A)	N/A	Contact residue to NF-κB disrupted	No	Positive
5780	Wildtype	N/A		No	Negative
6763	Wildtype	N/A		No	Positive
6214	Exon 1 (201 C>A)	N/A		Yes	Negative
6861	Exon 1 (213 T>A)	N/A	Non-conservative substitution	Yes	Positive
6766	Wildtype	N/A		No	Positive

Table 4.6 *IkBa* gene mutation and loss of heterozygosity (LOH) in 10 cases of cHL (N/A, not available).

Many of the cases (3 of 10 for REL and 8 of 10 for STAT3/5a) did not have centromere copy number data analysed by both observers. The DAPI staining of the nuclei often appeared fragmented and dim particularly in the HRS cells (see Figure 4.5). In cases where the DAPI staining was weak, there was weak or no FISH signals despite the presence of good surface immunostaining. To increase the sensitivity of the analysis in these cases the incubation with the primary antibody was extended to overnight instead of RT for 30 minutes, the time of the simultaneous denaturation of probes and DNA was extended from 7 to 12 minutes

and hybridisation time was lengthened by an extra 24 hours. Although satisfactory FISH signals were obtained in most cases, it was expected that not all signals were revealed given the fragility of the HRS cells. Imprint preparations were not superior in quality compared to cytospins. There were often more eosinophils on imprints leading to autofluorescence and difficulty in analyses.

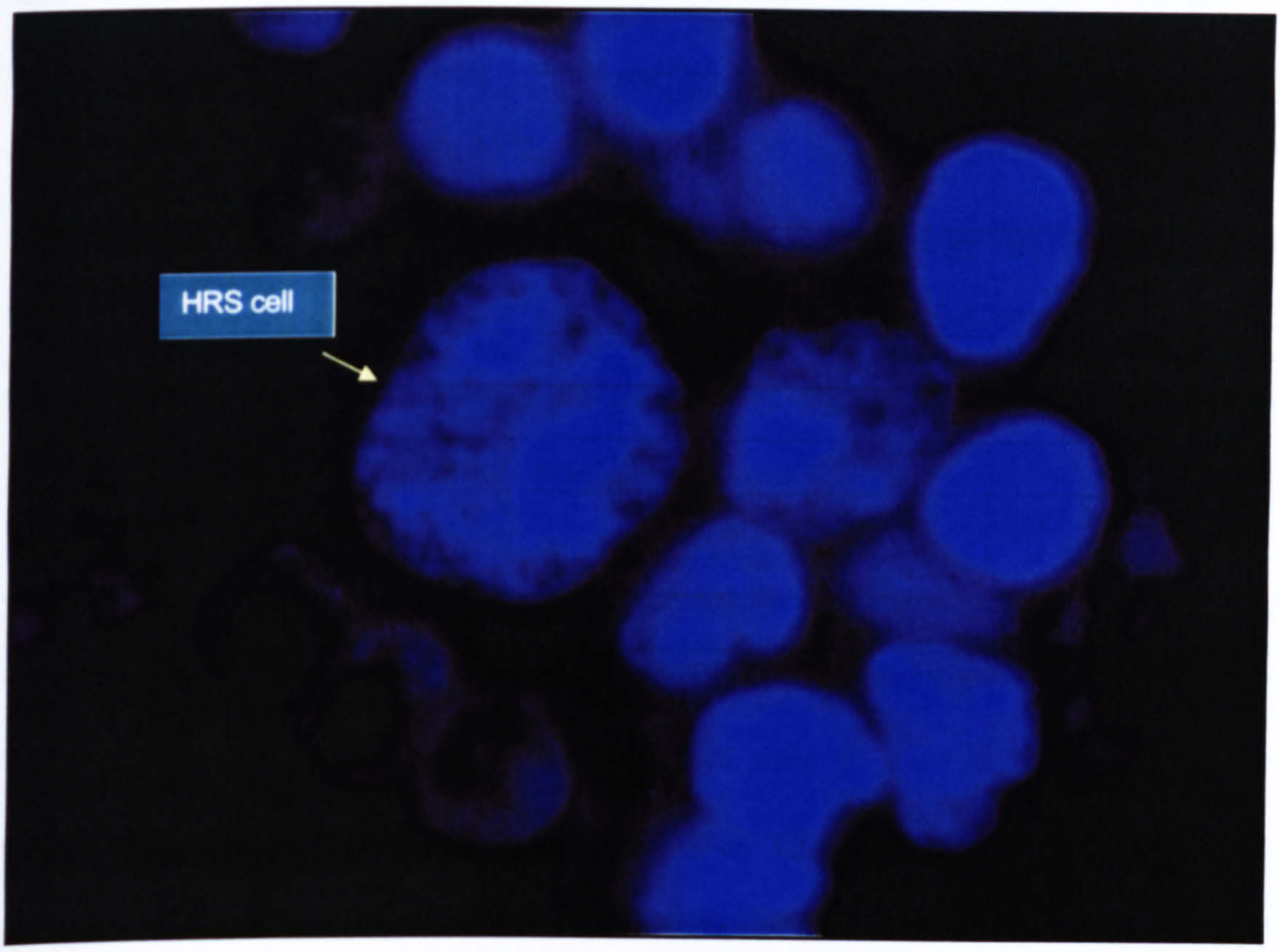

Figure 4.5 DAPI staining of HRS cells and surrounding infiltrate. The quality of DAPI staining was often less good in the HRS cells in comparison to adjacent lymphocytes, indicating their fragility. This image shows adequate DAPI staining in the HRS cell.

4.4 Discussion

In the present study, the frequency of REL and STAT3/5a gains in the CD30-positive HRS cells from 10 cHL cases was investigated using the FICTION technique. This allowed us to bypass the difficulty in obtaining tumour metaphases from cHL. Probes for the corresponding centromere were used (CEP 2: D2Z1 and CEP 17: D1721) to demonstrate chromosome copy number change in relation to the gene copy number. Results were collected by 2 independent observers and correlated to CGH data where available. There was only minor inter-observer variability and this was addressed by a third confirmatory evaluation. Although both observers evaluated the same slides, they might not have seen and recorded the same cells. This can have a profound effect especially when the HRS cells appear to have such a high level of genomic instability.

Martin-Subero et al. (2002) have observed a gain of REL in 35% to 55% of cHL cases analysed by FICTION with or without the adjustment for centromere 2 copy number respectively. In this study a gain of REL in 4 of 10 cHL cases was observed, which was in complete agreement with our CGH analyses where available. This provides further evidence that the gain of 2p in cHL detected by CGH is consistently associated with a gain in the REL gene.

The presence of LOH or mutation of the *IkBa* gene in these 10 cases of cHL was also investigated. Mutations occurred frequently with 6 out of 10 cases having one to three mutations, although only 3 cases harboured potentially functionally important mutations. LOH was also detected in 2 cases. No distinct pattern of

correlation between gains of REL by FICTION, EBV status, LOH and *IkBa* gene mutation status were observed among these cases. In one EBV-negative case (6689) where the clinical course of the disease was particularly aggressive, the HRS cells harboured both REL amplification and bi-allelic inactivating IκBα gene mutations. A dosage effect is possible but this would suggest that these two mechanisms are not mutually exclusive of each other. As REL amplifications are found in both EBV-positive and negative cases of cHL, this would indicate that c-rel and EBV are not mutually exclusive of each other. *IkBa* mutations are not present in all EBV-negative cases of cHL which suggests *IkBa* mutations are not substituting for EBV. In one EBV-negative case (5780) there was wild type *IkBa* and no REL amplification; this suggests the possibility of another mechanism leading to the activation of NF-κB in the HRS cells.

Our CGH data indicated frequent gain of 17q in cHL. From our FICTION data, 7 of 10 cases appeared to have numerical aberrations of chromosome 17 in at least some of the HRS cells. No bystander cells with such aberration were found by either observer. Although 5 of 10 cases appeared to have STAT3/5a copy numbers above the diploid range, this became insignificant when related to the centromere 17 copy number changes. Indeed, none of the 10 cases can be classified as having a gain of STAT3/5a nor is there any indication that translocation is a common event. There could be two reasons for such an observation. Firstly, additional signals of STAT3/5a were not detected due to the sub-optimal quality of DNA in the samples. Secondly, STAT3/5a is not involved in the gains observed on 17q by CGH analyses. This might indeed be the case as

many of the gains appeared to be near 17q12 or 17q24 by CGH analyses and STAT3/5a is not the only gene located on 17q21. The high level of STAT5a protein and RNA expression observed in the HL-derived cell line L428 may result from up-regulation of STAT5a by NF-κB rather than from STAT5a gene itself. Thus, activation of NF-κB can lead to both increased expression and activation of STAT5a (Hinz et al. 2002).

Our FICTION data have provided confirmation that the gain of 2p detected by CGH in cHL consistently involves REL but the gain in 17q is not associated with a gain in STAT3/5a. This suggests that other genes may be involved in 17q gains. Our data also show that activation of NF-κB by REL amplification, EBV, *IkBa* mutation or LOH are not mutually exclusive mechanisms.

Chapter 5

GENERAL DISCUSSION

It has taken well over 150 years, from the first description of HD / HL, to elucidate the origin of the tumour cells, the so called Hodgkin and Reed-Sternberg (HRS) cells. It is now recognised that the HRS cells, in most instances, are derived from germinal centre B cells. In addition, in approximately 30% of cases, the HRS cells are known to harbour EBV. They do not display surface Ig and have non-functional B cell receptor (BCR) transcription mechanisms. Our current understanding of B cell development suggests that such non-functional B cells should be eliminated by apoptosis. Yet these HRS cells can persist. Mechanisms used by HRS cells to survive may include the constitutive activation of NF-κB, disruption of the TRAF pathways, mimicry of survival signals by EBV LMP2 and LMP1 and suppression of immune surveillance by the recruitment of immunosuppressive T cells.

The scarcity of the HRS cells within tumours has hampered research significantly despite the expansion of scientific knowledge in molecular biology. As mentioned previously, cytogenetic studies have made enormous advances in many other tumours and lymphoma where consistent chromosomal abnormalities have led to the discovery of oncogenes and tumour suppressor genes. This has not been possible in cHL as most of the karyotypes are either normal, probably due to outgrowth of bystander cells, or so complex that no meaningful interpretation could be made. In this project, the problems have been overcome with the combination of several recent advances in molecular techniques, namely: isolation of single HRS cells by CD30 ICC and LMD; universal amplification of the small amount of DNA to usable quantity by DOP-PCR; and the identification of genomic

imbalances without the need for tumour metaphase spreads by CGH. The availability of the entire human genome sequence and FISH-mapped probes has also enabled further elucidation of candidate genes involved in the pathogenesis of cHL. Many of the techniques involved in this project were new to this laboratory. Much time and resources were therefore invested in trouble-shooting and establishing these as robust techniques in our hands.

Overall, we have been successful in establishing LMD of single HRS cells for all our downstream analyses including various PCRs and CGH. Analysis of 20 cases of cHL using CGH revealed recurrent chromosomal imbalances. The most frequent abnormality was gain on 17q (70%). Other frequent gains involved 2p (40%), 12q (40%), 17p (40%), 22q (35%), 9p (30%), 14q (30%) and 16p (30%). The most frequent losses involved 13q (35%), 6q (30%), 11q (25%) and 4q (25%). The data strongly resembles those generated from the Heidelberg group despite using different protocols and patient samples (Ohshima et al. 1999; Joos et al. 2000; Joos et al. 2002). In the present study, HRS cells were obtained by LMD from cytospin preparations that had been immunostained using CD30 monoclonal antibodies. This method of collection of HRS cells offers advantages over previous methods used in CGH experiments in that single HRS cells were individually selected using stringent criteria, and the use of cytospins prepared from viable material ensured that complete cells were captured, hence the robust nature of this study. Results were also analysed with respect to clinicopathological parameters. Although the number of cases analysed was small and statistical power limited, these analyses provide hypotheses for further testing. There were

significantly more gains on 2p and 14q in the older adult cases (p-value = 0.038 and 0.022 respectively); and losses of 13q were associated with a poor clinical outcome (p = 0.049). This study also provided further evidence that cHL is genetically distinct from NLPHL. Apart from the gain of 12q and loss of 11q, there are no shared abnormalities with the 20 chromosomal imbalances described in NLPHL by Franke et al. (2001). One observation that is common amongst all the CGH studies in HL is the unusually high number of chromosomal abnormalities observed. This may indicate an underlying mechanism for genomic instability with a recurring pattern in HL, as discussed previously and recently (Mark et al. 1998; Re et al. 2002; Joos et al. 2003).

The chromosomal imbalances detected in cHL are different from those detected in most other lymphomas with the exception of primary mediastinal B-cell lymphoma (PMBCL). PMBCL is the only NHL which shares frequent gains of 2p and 9p and losses of 13q, 6q and 4q (Bentz et al. 2001; Palanisamy et al. 2002). This is of particular interest since both tumours have a tendency to occur in young females, have a predilection for the mediastinum, and lack expression of surface Ig.

Gain of chromosome 17q was the most frequent abnormality found in this CGH study of cHL. Alteration of 17q was also one of the more frequently found abnormalities in previous cytogenetic studies of cHL (Sarris et al. 1999). Many genes that have been previously implicated in cHL, including genes involved in, or regulated by, NF-κB activation, and genes encoding chemokines and STATs reside in 17q. As part of my follow-up study, I investigated the frequency of REL

and STAT3/5a gains in the CD30-positive HRS cells from 10 cHL cases using the FICTION technique. In this study a gain of REL in 4 of 10 cHL cases was observed, which is in complete agreement with my CGH analyses where available. This provides further evidence that the gain of 2p in cHL detected by CGH is consistently associated with a gain in the REL gene. Our data also show that activation of NF-κB by REL amplification, EBV, *IkBa* mutation or LOH are not mutually exclusive mechanisms.

From our FICTION data, 7 of 10 cases appeared to have numerical aberrations of chromosome 17 in at least some of the HRS cells. None of the 10 cases would be classified as having a gain of STAT3/5a nor was there any indication that translocations involving these genes are a common event. Trisomy 17 leading to the frequent gains observed by CGH cannot be ruled out but the findings could also be suggesting the importance of other candidate genes present in 17q. In addition to those already mentioned in Chapter 3, a recent paper highlighted a novel chromosomal derrangement mechanism in cHL associated with proteins involved in cell division, namely pericentrin and dynein which are both located in 17q (Martin-Subero et al. 2003).

There are several ways in which this current project could be further developed exploring different aspects of the biology of cHL. FICTION could be used to look for other candidate genes in 17q or other areas of frequent amplification detected by CGH. SNP assays could be used to look for LOH in deleted regions which may harbour unknown tumour suppressor genes. FICTION could also be used simply

to delineate numerical chromosomal abnormalities with particular reference to chromosome 17. Another major area that would merit further study would be the mechanism leading to such profound chromosomal instability in the HRS cells, whether at a genetic or epigenetic level.

A high throughput, high resolution microarray-based comparative genomic hybridisation (M-CGH) has recently been introduced. This technique provides a superior spatial resolution, allowing a fully automated evaluation of experiments and a simultaneous analysis of hundreds of genomic loci. In contrast to chromosomal CGH, the array-based genome screening has a 20-100 fold higher resolution (Wessendorf et al. 2002). The major limitation of this method is that it requires genomic DNA and experiments using DOP-PCR amplified DNA have been inconsistent. This problem might be overcome with a different method of WGA. Rolling circle amplification using φ29 DNA polymerase and random exonuclease-resistant primers was developed for amplifying large circular DNA templates such as plasmid and bacteriophage DNA. Rather unexpectedly, these reagents also readily amplify linear, human genomic DNA in a cascading, strand displacement reaction termed multiple displacement amplification (MDA). Amplification of genomic DNA by MDA leads to a large amount of product with an average product length exceeding 10 kb. The yield of MDA was also found to provide more complete coverage of the genome with less amplification bias when compared with PEP-PCR and DOP-PCR (Dean et al. 2002). MDA also compared favourably with DOP-PCR for CGH, and suppression hybridisation may be unnecessary for detection of single copy sequences. This makes it particularly

attractive for chip-based genetic analysis from limited patient DNA (Dean et al. 2002; Lovmar et al. 2003). At the present time, MDA is not optimised for small starting amounts of DNA template (our unpublished data) but this technique holds promise for the future. The use of M-CGH should further refine the recurrent genetic imbalances, which are a feature of cHL, and help to identify key genes in cHL pathogenesis.

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APPENDIX

Materials

Material	Source
0.2 ml PCR tubes	Applied Biosystems, UK
Agarose powder	Invitrogen Ltd., Paisley, UK
Apparatus for agarose gel electrophoresis	BRL, Paisley, UK
Apparatus for polyacrylamide gel electrophoresis	Bio-Rad Laboratories, Hertfordshire, UK
β-mercaptoethanol	Sigma-Aldrich Company Ltd., Poole, UK
Bovine serum albumin (nuclease free)	Sigma-Aldrich Company Ltd., Poole, UK
CGH hybridisation buffer	Abbott Laboratories, Berkshire, UK
CoT-1 DNA, human	Invitrogen Ltd., Paisley, UK
D.P.X.	BDH, Poole, UK
DAPI II	Abbott Laboratories, Berkshire, UK
dATP, dCTP, dGTP and dTTP (100 mM)	Amersham Bioscience, Buckinghamshire, UK
Dimethylsulphoxide DMSO	Sigma-Aldrich Company Ltd., Poole, UK
DNA polymerase 1	Invitrogen Ltd., Paisley, UK
dUTP-SG (1 mM)	Abbott Laboratories, Berkshire, UK
dUTP-SR (1 mM)	Abbott Laboratories, Berkshire, UK
Falcon tubes (15 ml, 50 ml)	Becton Dickinson, Cowley, UK
Fliptop microcentrifuge tubes (1.5 ml)	Scotlab, Strathclyde, UK
FBS	Invitrogen Ltd., Paisley, UK
Petri dish	Scotlab, Strathclyde, UK
Phenol/ chloroform/ isoamylalcohol (PCI9)	Sigma-Aldrich Company Ltd., Poole, UK
Pipette tips (Rainin)	Scotlab, Strathclyde, UK
Proteinase K	Sigma-Aldrich Company Ltd., Poole, UK
Tryptone peptone	Sigma-Aldrich Company Ltd., Poole, UK
Tween 20	Sigma-Aldrich Company Ltd., Poole, UK
Wide-bore polypropylene pastette	Alpha Laboratories, Hampshire, UK
Yeast extract	Sigma-Aldrich Company Ltd., Poole, UK

Buffers and solutions

	Recipe
10x loading buffer for non-denaturing gel electrophoresis	Bromophenol blue 0.42% Xylene cyanol 0.42% Glycerol 50%
20x sodium chloride and sodium citrate (SSC) (Store at room temperature discard after 6 months)	66 g 20xSSC mix (Abbott Laboratories) in 200 mls of dH_2O Adjust to pH 5.3 using concentrated HCl Adjust volume to 250 ml using dH_2O Filter through a 0.45 um filter
A4 mixture	0.2 mM dATP 0.2 mM dCTP 0.2 mM dGTP 500 mM Tris-HCl (pH 7.8) 50 mM $MgCl_2$ 100 mM β-mercaptoethanol 100 µg/ml BSA (nuclease free).
Antifade solution	230 mg of DABCO 10 ml of PN buffer (see below) 90 ml of glycerol
Cell culturing medium (10% FBS)	500 ml RPMI 1640 with L-Glutamine (Invitrogen) 50 ml Fetal Bovine Serum (FBS) (Invitrogen) 20 ml Penicillin / Streptomycin (Invitrogen) 5 ml L-Glutamine (Invitrogen)
Column buffer (Sephadex G-50)	2.5 ml 1 M Tris-HCl, pH 8 0.5 ml 0.5 M EDTA 2.5 ml SDS 10% Fill up to 250 ml with dH_2O
DAPI solution	6 µl of DAPI stock solution 60 ml of 2xSSC
DAPI stock solution	0.2 mg/ml of DAPI in dH_2O
Denaturation solution (Prepare in a fume hood on the day of use)	35 ml formamide 5 ml 20x SSC 10 ml dH_2O Adjust pH to 7.0 – 7.5

Freezing medium	92% fetal calf serum (Invitrogen) 8% DMSO (Sigma)
Hydrogen peroxide/ methanol solution (1.5%) (Discard within 1 month)	3 ml hydrogen peroxide 57 ml methanol
Luria-Bertani (LB) medium for growing BACs (Solid LB medium was prepared in the same way with the addition of 15 g/L agar and sterilised by autoclaving). (Media were supplemented with chloramphenicol (20 μg/ml) as the BACs used were chloramphenicol resistant).	10 g of tryptone-peptone 5 g of yeast extract 5 g of NaCl Make up to 900 ml with dH_2O Adjusted pH to 7.4 Fill up to 1000 ml with H_2O Aliquoted and sterilised in autoclave
Master mix for single copy probes	5 ml deionized formamide 2 ml dextran sulphate 50% 1 ml 20xSSC
Mini-Macs buffer	50 ml 10x PBS 10 ml FBS 2 ml EDTA 2.5 g BSA Fill to 500 ml with dH_2O Filter and aliquot
Nick translation enzyme mix	0.4 U/μl Pol1 40 pg/μl DNase I
Paraformaldehyde (1%)	Paraformaldehyde 1 g in 60 ml of dH_2O Add 5 drops of 10 M NaOH Heat the solution until transparent Allow to cool Add 10 ml of 100 mM $MgCl_2$ Adjust pH to 7.0 – 7.5 Fill up to 100 ml with dH_2O Filter the solution
PBS	137 mM NaCl 2.7 mM KCl 10 mM Na_2HPO_4. 2 mM KH_2PO_4.

Pepsin digestion solution	0.5 ml of 2 M HCl 99.5 ml of dH_2O Add 5 mg of pepsin
PN buffer	0.1 M 13.8 g $NaH_2PO_4.2H_2O$ *0.1 M 17.8 g $Na_2HPO_4.2H_2O$* Add dH_2O to 900 ml. Adjust pH to 8.0 and add dH_2O to 1 L.
PNM buffer	Add 5 g of milk powder to 100 ml of PN buffer Heat to 50°C and leave stirring overnight Add 0.03 g of NaN_3 Centrifuge and use supernatant only Keep at 4°C for 6 months
Sephadex G-50 suspension	10 g Sephadex G-50 + 160 ml of dH2O Leave to stand for 30 minutes at RT Centrifuge at 400 g for 4 minutes at RT Remove supernatant Wash Sephadex twice with dH_2O at 400 g Leave at 3:1 solid to aqueous phase
SpectrumRed or SpectrumGreen (SR/SG) 1 mM mix	1:1 dTTP: fluorescent labelled dUTP
TAE	40mM Tris 20mM Sodium acetate 20mM Sodium chloride 2 mM EDTA Adjusted to pH 8
TaqMan lysis buffer	25 mM Tris, pH 8.8 0.01 mM EDTA 0.45% Tween 20 0.45% NP40
TBE	90 mM Tris 90 mM Boric acid 2.25 mM EDTA Adjusted to pH 8

TBS	50 mM Tris HCl *0.05 M Tris base* *20 mM NaCl* Adjusted to pH 7.6
TBST	100 ml 20xTBS with 0.1% Tween 20 1900 ml of dH_2O
TE	10 mM Tris pH 8 1 mM EDTA pH 8
ThermoSequenase lysis buffer	1xTSB (260mM Tris-HCl pH 9.5, 65mM $MgCl_2$) 0.25 mg/ml Proteinase K 0.45% Tween 20 0.45% NP40
TNE	0.1mM Sodium chloride 10mM Tris 1mM EDTA Adjusted to pH 8
Travel medium	500 ml RPMI1640 with L-glutamine 100 ml Fetal calf serum 20 ml Penicillin/Streptomycin 5 ml L-glutamine 5 ml Fungizone 7.5 ml Gentamicin 12.5 ml Hepes buffer
Wash Buffer 1 (0.4x SSC) (Store at room temperature and discard after 6 months)	10 ml 20xSSC 1.5 ml NP40 475 ml dH_2O Adjust pH to 7.0 – 7.5 with NaOH Fill up to 500 ml Filter through a 0.45 μm filter
Wash Buffer 2 (2x SSC) (Store at room temperature and discard after 6 months)	50 ml of 20xSSC, 0.5 ml NP40 425 ml dH_2O Adjust pH to 7.0 – 7.5 with NaOH Fill up to 500 ml Filter through a 0.45 μm filter.

Cell lines

Cell lines	Information	EBV status
Raji	ATCC CCL-86 Burkitt's lymphoma, human Male Chromosome: 2n = 46	Positive
Daudi	ATCC CCL-213 Burkitt's lymphoma, human Male Chromosome: 2n = 46	Positive
IM-9	ATCC CCL-159 Lymphoblastoid cell line, human Female Chromosome: 2n = 46	Positive
KM-H2	DSMZ ACC 8 Hodgkin lymphoma, human MCHL > LDHL, stage IV Male Chromosome: hypotriploid karyotype with polyploidy	Negative
L428	DSMZ ACC 197 Hodgkin lymphoma, human NSHL, stage IV B Female Chromosome: hypertetraploidy karyotype with polyploidy	Negative
L591	Kindly provided by Dr. David Jones, University of Southampton Hodgkin lymphoma, human NSHL, stage IV Female Chromosome: not available	Positive
L1236	DSMZ ACC 530 Hodgkin lymphoma, human MCHL, stage IV Male Chromosome: hypotriploid karyotype with polyploidy	Negative

IkBa primers

ID	Primer Name	Primer Sequence
699	Exon 1, forward outer	TGGTCTGACTGGCTTGGAAATTC
720	Exon 1, reverse outer	GCGTCCCGCCCTCCCGACGA
593	Exon 2, forward outer	CCTCTCTTCCCCACAGGTTCCT
702	Exon 2, reverse outer	AAAGGATCTGGGGTGACTCT
721	Exon 3, forward outer	CCTGTCTAGGAGGAGCAGCAC
694	Exon 3, reverse outer	AAAGGCATCCAATAGGCAC
722	Exon 4, forward outrer	GAACCCAGACTGTGGGTTCT
696	Exon 4, reverse outer	TGAGATGCTTATGGCTGCA
724	Exon 5, forward outer	ATGCTCAGGTTGGTGCTTCC
726	Exon 5, reverse outer	CTGGGAGGGTGAAGGGAAT
703	Exon 6, forward outer	CCCATCCCGGTAGCTTGGCAG
708	Exon 6, reverse outer	TTCAGTGATGTGGGGTGAAA
746	Exon 1, forward inner	AGCGCCCCAGCGAGGAAGCA
719	Exon 1, reverse inner	TCGGTGAGCTGCTGCTTCCA
745	Exon 2, reverse inner	ATCAGCTACGTCCCAGGGTC
684	Exon 3, forward.inner	AGGAGACACGGGTTGAGG
723	Exon 4, forward inner	AGGTGAAAGGAGTGAGGGTTG
725	Exon 5, forward inner	GCACTGAGTCAGGCTCCTCG
627	Exon 6, reverse.inner	GGATACCACTGGGGTCAGTCACTC

Primers 593, 694, 696, 726 and 703 were used in both first and second round PCRs.