INVESTIGATION OF THE AETIOLOGY OF HODGKIN'S DISEASE

by

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LIST OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

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DECLARATION

I declare that the content of this thesis unless otherwise stated is entirely my own work.

SUMMARY

Hodgkin's disease (HD) is a malignant lymphoma characterised by the presence of the Reed Sternberg (RS) cell, the proposed malignant cell of the disease, in a polymorphous cellular background. The aetiology of HD is unknown and is the subject of this thesis. The RS cells account for around 1% of the tumour mass and therefore characterisation of the RS cell has proven difficult. Epstein-Barr virus (EBV) has been detected in and localised to the RS cells in around 40% of HD tumours. The EBV latent membrane protein 1 (LMP-1) is expressed by the RS cells in EBV-positive cases. LMP-1 has been shown to up-regulate the B-cell lymphoma-2 (*bcl*-2) proto-oncogene and increased Bcl-2 expression is known to protect cells from apoptosis or programmed cell death. In addition the t(14;18) translocation, which also causes increased Bcl-2 expression, had been reported previously in HD. We investigated a series of HD cases for the presence of the t(14;18) translocation utilising a PCR technique. In a proportion of cases a comparison between LMP-1 and Bcl-2 expression was made. We found few translocations present in the HD cases examined and in addition no correlation was observed between LMP-1 positivity and Bcl-2 expression. We conclude that the t(14;18) translocation is an infrequent finding in HD and is unlikely to play an aetiological role in this malignancy.

An animal model for the study of HD would aid in studies such as that described above. The use of severe combined immunodeficient (SCID) mice for the study of human malignancies has been well documented. We attempted to engraft HD-derived tumour tissue into SCID mice in order to propagate RS cells for the subsequent investigation of viral and oncogene involvement in this disease. In addition tumour cell suspensions from non-Hodgkin's lymphoma (NHL) samples were also transplanted into SCID mice. The transplantation of NHL tumour cell suspensions was a success, with a proportion of tumours in SCID mice showing identical genotype and phenotype to original biopsy material. The transplantation of HD tumour material into the SCID model was however less successful with SCID tumours showing an EBV-driven lymphoproliferative phenotype indicating that outgrowth of EBV-infected bystander cells in the tumour had occurred. At present the SCID model appears an unsuitable system for the propagation of the RS cells of HD.

HD has a bimodal age incidence curve with a peak incidence in young adults; the cases within this peak particularly those of the nodular sclerosis subtype are infrequently EBV-associated. Epidemiological studies suggest that an infectious agent may be involved in the aetiology of the young adult disease. Candidate infectious agents are herpesviruses since they are widespread in nature and establish persistent infection. We devised a PCR strategy for the detection of herpesvirus sequences in DNA samples using primers based on two well-conserved regions of the herpesvirus glycoprotein B gene. The assay has sufficient sensitivity to detect herpesvirus sequences if present within the RS cells of a HD biopsy specimen. The assay also has the capability of distinguishing between different herpesviruses within a given sample. Use of a technique such as this may show that another herpesvirus is involved in the pathogenesis of the non EBV-associated cases of HD.

ABBREVIATIONS

Ag	antigen
AIDS	acquired immunodeficiency syndrome
AILD	angioimmunoblastic lymphadenopathy
Bcl-2	B-cell lymphoma 2
BHV	bovine herpesvirus
BL	Burkitt's lymphoma
C	constant region
c^7dGTP	7-deaza-2' deoxyguanosine triphosphate
D	diversity region
DNA	deoxyribonucleic acid
dsDNA	double-stranded DNA
EBERs	EBV-encoded RNAs
EBNAs	EBV nuclear antigens
EBV	Epstein-Barr virus
EHV	equine herpesvirus
FDRCs	follicular dendritic reticulum cells
FHV	feline herpesvirus
Fig	Figure
gB	glycoprotein B
HCMV	human cytomegalovirus
HD	Hodgkin's disease
HHV	human herpesvirus
HIV	human immunodeficiency virus
HMW	high molecular weight
HPV	human papilloma virus
HSV-1	herpes simplex virus 1
HSV-2	herpes simplex virus 2
Ig	immunoglobulin
IgH	Ig heavy chain
ILTV	infectious laryngotracheitis virus
IM	infectious mononucleosis
J	joining region
LCL	lymphoblastoid cell line
LDHD	lymphocyte depleted HD
LMP	latent membrane protein
LPHD	lymphocyte predominance HD
mbr	major breakpoint region
MCHD	mixed cellularity HD
MCMV	murine cytomegalovirus
MDV	Marek's disease virus
Mg	magnesium
NHL	non-Hodgkin's lymphoma
No.	number
NSHD	nodular sclerosis HD
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction

PRV	pseudorabies virus
RNA	ribonucleic acid
RS	Reed-Sternberg
SCID	severe combined immunodeficient
SDS	sodium dodecyl sulfate
SHV	Simian herpesvirus
TCR	T-cell receptor
UNPC	undifferentiated nasopharyngeal carcinoma
V	variable region
VNTRs	variable number of tandem repeat sequences

CHAPTER 1
GENERAL INTRODUCTION

1.1. CLASSIFICATION OF LYMPHOMAS

Human malignant lymphomas are broadly divided into two groups- Hodgkin's disease (HD) and the non-Hodgkin's lymphomas (NHLs) which can be further divided into B- and T-cell lymphomas (Stansfeld *et al.*, 1988). Several systems for the classification of lymphomas are in current use and are a matter of some controversy (Lukes & Collins, 1975; Rosenberg, 1982; Bennett *et al.*, 1991). Ideally, lymphomas should be classified according to their presumed normal counterpart, however our current knowledge does not permit reliable classification on this basis (Harris *et al.*, 1994). In contrast to the NHLs, the Rye classification system for the subdivision of HD (Lukes *et al.*, 1966) has remained stable and is generally accepted as pathologically relevant (Bennett *et al.*, 1991).

1.2. HODGKIN'S DISEASE

HD was first described in 1832 after a study involving the examination of seven lesions of the lymphatic glands and spleen (Hodgkin, 1832). The term Hodgkin's disease was applied some 30 years later following examination of cases for clinical and gross pathological findings (Wilks, 1865). The histology of HD was first described by Sternberg in 1898 and detailed further by Reed in 1902 (Sternberg, 1898; Reed, 1902). Both authors described the large bilobed or multinucleated giant cells now termed Reed-Sternberg (RS) cells which are characteristic of the disease. Both authors believed that the disease was infectious in nature; Sternberg proposed a Mycobacterial infection and Reed some other unknown agent.

1.2.1. Clinical features of HD

HD most commonly presents with lymphadenopathy, often accompanied by constitutional 'B' symptoms including, tiredness, weight loss, fever and night sweats (Selby & McElwain, 1987). Common sites of lymphadenopathy at presentation are the neck and mediastinal lymph nodes (Mauch *et al.*, 1993).

1.2.2. Histopathological features of HD

The diagnosis of HD is based on the recognition of the proposed malignant cell, the RS cell or morphological variant, present in a polymorphous cellular background (Lukes & Butler, 1966; Lukes, 1971). The cellular infiltrate is heterogeneous consisting of lymphocytes, histiocytes, granulocytes including eosinophils, plasma cells, and fibroblasts. The presence of the RS cell within an appropriate cellular background is necessary for the diagnosis of HD as RS-like cells are also found in other conditions (Strum *et al.*, 1970).

1.2.3. Histopathological classification of HD

There is a range of histological appearances in HD and on this basis the disease has been divided into four subtypes: nodular sclerosis (NSHD), mixed cellularity (MCHD), lymphocyte depleted (LDHD) and lymphocyte predominance (LPHD). The earlier classification system of Lukes & Butler further subdivided the LDHD and LPHD categories as described in sections 1.2.3.3. and 1.2.3.4. The recent "REAL" lymphoma classification system suggests the inclusion of the provisional disease entity lymphocyte-rich classical HD in order to differentiate LPHD, as described below, from classical HD with abundant lymphocytes (Harris *et al.*, 1994). It is now recognised that LPHD is a distinct entity that should be classified separately from the other subtypes (Nicholas *et al.*, 1990; Mason *et al.*, 1994).

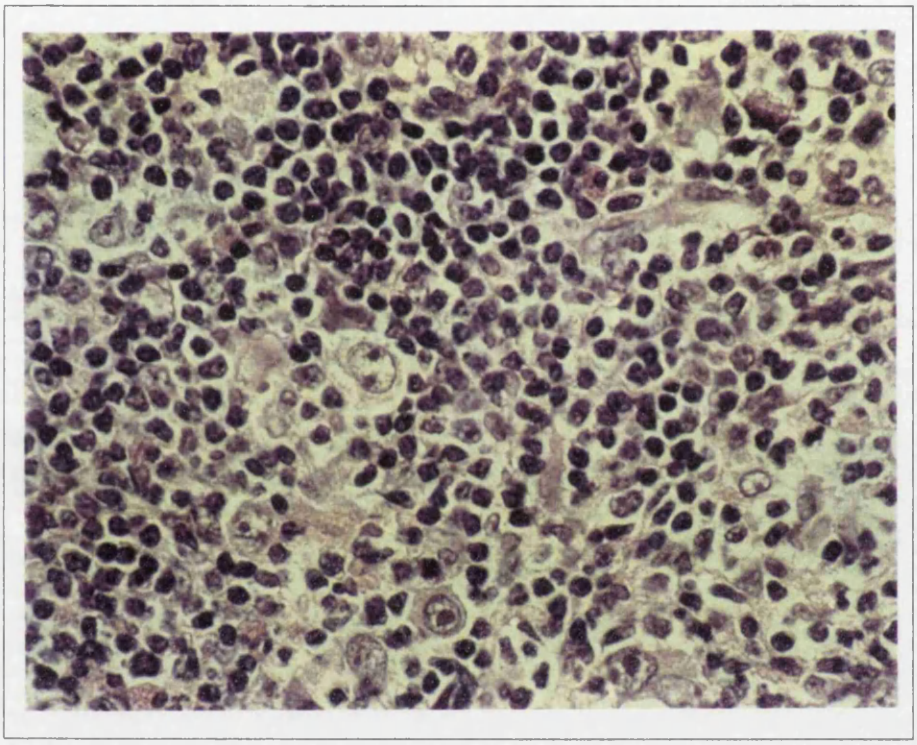
1.2.3.1. Nodular sclerosis HD

NSHD occurs mainly in young adults and in contrast to the other subtypes of the disease does not show a male predominance. NSHD accounts for 70% of cases and therefore is the most common histological subtype. The diagnosis relies on the histological detection of classical RS cells in a polymorphous background including lacunar cells. There is prominent fibrosis with collagen bands dividing the node into nodules (Bennett *et al.*, 1991) (Fig 1.1.). The disease has been further divided

Fig 1.1. Haematoxylin and Eosin stained section from a case of NSHD (x 100 magnification).

Photograph kindly provided by Dr. A.S. Krajewski (Department of Pathology, University of Edinburgh).

Fig 1.1. Stained section of a NSHD case



into good and poor prognostic categories termed NS-1 and NS-2 respectively (MacLennan *et al.*, 1989).

1.2.3.2. Mixed cellularity HD

MCHD occurs mainly in young adults, however it is also the most common subtype found in older patients and shows a male predominance. It accounts for between 20 and 40% of cases and is therefore the second most common subtype of the disease (Colby *et al.*, 1982). The number of RS cells and the reactive cellular infiltrate in this subtype can be widely variable. Criteria for inclusion in this category are ill-defined as cases which do not fulfil the definition of NSHD or LPHD tend to be classified as MCHD. The cellular background may range from predominantly lymphocytic to lymphocyte depleted, although eosinophils, plasma cells and histiocytes are generally also observed. RS cell variants may be present and in addition areas of irregular sclerosis are sometimes observed (Fig 1.2.).

1.2.3.3. Lymphocyte depleted HD

LDHD can be sub-divided into two groups: diffuse fibrosis and reticular (Lukes & Butler, 1966). In present day studies this subtype accounts for less than 5% of cases and is therefore the least common (Kant *et al.*, 1986). Diagnosis of the diffuse fibrosis subgroup is dependent on the detection of few classical RS cells and the presence of numerous large mononuclear variants of RS cells in a depleted cellular background. In the reticular subtype small numbers of lymphocytes, neutrophils and eosinophils are present along with cellular sheets of RS cells containing bizarre nuclei and mononuclear cells. Both subtypes are commonly observed within the same biopsy specimen.

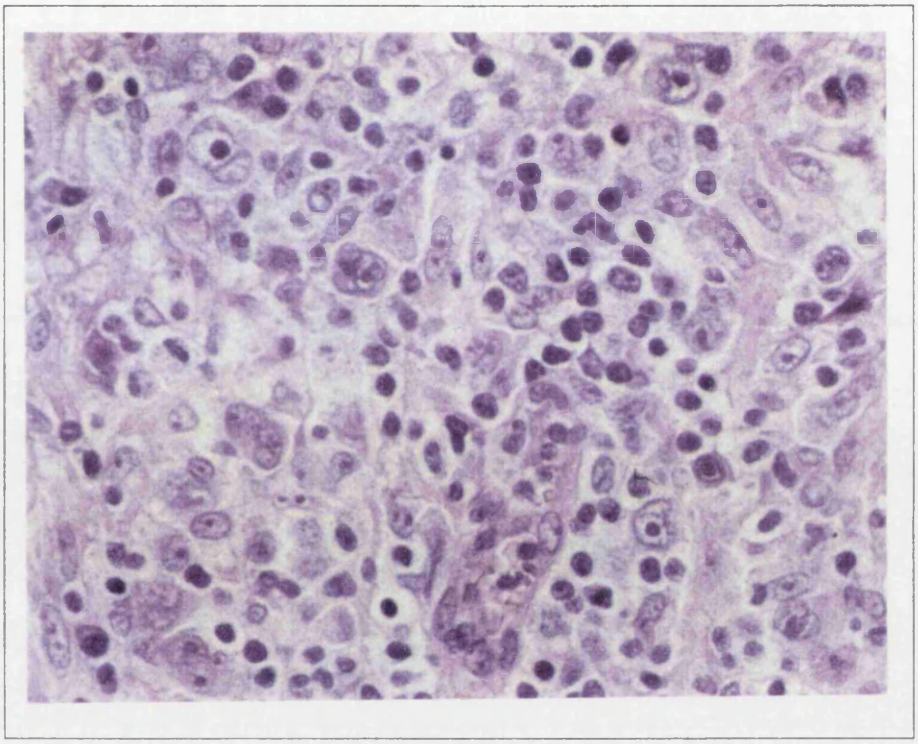
1.2.3.4. Lymphocyte predominance HD

LPHD occurs at all ages and shows a marked male predominance. It accounts for around 5-10% of cases and is divided into nodular

Fig 1.2. Haematoxylin and Eosin stained section from a case of MCHD (x 250 magnification).

Photograph kindly provided by Dr. A.S. Krajewski (Department of Pathology, University of Edinburgh).

Fig 1.2. Stained section of a MCHD case



lymphocytic and histiocytic predominance HD (NLPHD) and diffuse lymphocytic and histiocytic predominance HD (DLPHD) (Lukes & Butler, 1966). This subgroup is characterised by the presence of L&H type RS cells that have multi-lobed nuclei and nucleoli that are less conspicuous than those of classical RS cells. These cells are often referred to as "popcorn" cells. Few classical RS cells are present in this subtype (Wright, 1989; Butler, 1992). The distinctive phenotype of L&H cells suggests that they are of B-cell origin (Poppema *et al.*, 1979a, 1979b; Stein *et al.*, 1986). These cases are now described as a distinct clinical entity (Nicholas *et al.*, 1990; Mason *et al.*, 1994).

1.2.4. Immunophenotype and genotype of RS cells

Immunophenotypic and genotypic studies in HD have been hampered by the relatively small proportion (often less than 1%) of RS cells within the tumour mass. Many studies have investigated the immunophenotype of RS cells using frozen and, in more recent years, paraffin sections from tumour biopsy material (reviewed by Hugh and Poppema, 1992; Drexler, 1992). Activation-associated antigens such as CD71 (transferrin receptor), and CD25 (interleukin 2 receptor) have consistently been shown to be expressed in RS cells (Hugh and Poppema, 1992). Adhesion molecules have also been demonstrated, e.g., CD54 (ICAM-1), CD58 (LFA-3), CD44 and CD15 (Paietta, 1992). Members of the nerve growth factor receptor family, CD30 and CD40 are also expressed (Durkop *et al.*, 1991; O'Grady *et al.*, 1994). CD30 is accepted as a reliable marker for the identification of classical RS cells.

The lineage of the classical RS cell is still unresolved. Inconsistent results have been obtained in studies using limited numbers of B- and T-cell markers on sections of paraffin-embedded tumour material (Hall *et al.*, 1988; Hugh & Poppema, 1992). In more detailed studies which employed the use of extended antibody panels, B-cell antigens were shown to be expressed by some of the RS cells in a large proportion of NSHD and MCHD cases (Schmid *et al.*, 1991). In contrast, other studies have shown that T-cell and not B-cell markers are expressed by RS cells in many cases of NSHD and MCHD (Agnarsson & Kadin, 1989; Dallenbach & Stein, 1989). The use of a novel technique involving the

micromanipulation of single RS cells has allowed the study of gene expression within these cells (Trumper *et al.*, 1993). The results from this study demonstrated that the pattern of mRNA expression was consistent with that of an activated haematopoeitic cell. The co-expression of genes characteristic for different cell lineages however did not allow specific definition of the cellular origin of RS cells.

The use of T-cell receptor (TCR) and immunoglobulin (Ig) gene rearrangements as a marker of clonal proliferation is discussed in section 4.1. The analysis of TCR and Ig gene rearrangements in HD has been inconclusive. The proportion of cases with detectable rearrangement of Ig genes varies from 0-25% between studies (Knowles *et al.*, 1986; Griesser *et al.*, 1987; Herbst *et al.*, 1989; Gledhill *et al.*, 1990). Studies which have selected cases on the basis of high RS cell numbers or enriched for RS cells have shown a higher proportion of rearrangements (Sundeen *et al.*, 1987; Weiss *et al.*, 1986). In several studies, the Ig genes in lesions containing large RS cell numbers were found to be in the germline configuration, clearly demonstrating that Ig gene rearrangements are not present in all cases. The results of TCR analysis on HD specimens are equally controversial. In two reports a high incidence of TCR rearrangements was documented (Griesser *et al.*, 1987; Herbst *et al.*, 1989) however other studies failed to confirm these findings (Gledhill *et al.*, 1990; Schmid *et al.*, 1991). These studies lend some support to the idea that RS cells are derived from lymphoid cells. There is also evidence suggesting that these cells may be derived from follicular dendritic reticulum cells (FDRCs) (Delsol *et al.*, 1993; Soderstrom *et al.*, 1994). In the first study CD21 expression was detectable in the RS cells of a proportion of cases. This antigen is also present in FDRCs. In the second study the FDRC marker, the acid cysteine proteinase inhibitor was detectable in RS cells in 88% of HD cases examined. Further studies are required to resolve this issue

1.2.5. Epidemiology of HD

The majority of studies show a bimodal age incidence curve for HD although the age incidence peaks occur in different age groups in different situations (MacMahon, 1966). These differences have led to the

suggestion that at least three epidemiological patterns of HD exist (Correa & O'Conor, 1971).

In developing countries the first peak of the bimodal curve occurs in childhood, there is a decrease in incidence in the third decade and a second peak occurs in older age groups. In developed countries, there is a low incidence in childhood but this increases with age resulting in a pronounced peak in young adulthood (15-34 years). The incidence declines in the fourth decade and in older years a gradual increase or plateau is observed (McKinney *et al.*, 1989; Glaser & Swartz, 1990). An intermediate pattern is found in rural areas of developed countries, in central Europe and in the southern United States (Alexander *et al.*, 1991a; Correa & Conor, 1971).

NSHD has a unimodal age incidence and accounts for the young adult peak seen in developed countries. In childhood, NSHD is relatively less common. The other subtypes as a group show a gradual increasing incidence with age which is a similar pattern to that observed for other lymphomas (McKinney *et al.*, 1989; Glaser & Swartz, 1990). There is a male predominance which is most pronounced in children and older adults and there is evidence that female cases in the young adult group are increasing (McKinney *et al.*, 1989; Glaser & Swartz, 1990).

Several hypotheses have been suggested to explain the epidemiological features of HD. MacMahon proposed that HD is a heterogeneous condition comprising at least three subgroups defined on the basis of age at onset of disease (MacMahon, 1966). He defined the three age groups 0-14 years, 15-34 years and 50 years and over and suggested that the three groups had different aetiologies. Since that time epidemiological studies have confirmed that the risk factors for the young adult and older age groups differ giving rise to a "two disease" hypothesis (Gutensohn, 1982; Alexander *et al.*, 1991a).

An alternative, but not mutually exclusive, hypothesis suggests that HD results from delayed exposure to a common infectious agent. This is known as the delayed exposure hypothesis or late host response model. Epidemiological studies have shown an increased risk of developing HD in individuals with a high socio-economic status in childhood (Alexander

et al., 1991b). It has been suggested that such persons have lacked early social contact and have therefore escaped infection with common childhood infections until later in life (Gutensohn & Cole, 1977). An extension of this hypothesis is the polio model which equates HD with paralytic polio in the prevaccine era (Gutensohn & Cole, 1981). This model suggests that like paralytic polio, HD in childhood in developing countries and young adult cases in developed countries are caused by the same infectious agent(s). These hypotheses are discussed further in section 1.4.1.1.

1.2.6. Aetiology of HD

The aetiology of HD remains an enigma. Studies of viral and oncogene involvement in HD have been hindered by the paucity of the RS cells within the tumour mass. The propagation of RS cells *in vitro* for the subsequent investigation of the malignancy has been relatively unsuccessful and as yet no animal model is available for the study of HD.

In recent years the human herpesvirus Epstein-Barr virus (EBV) has been detected and localised to the RS cells in a proportion of HD cases. The significance of these findings is discussed in detail in section 1.4.1. and the investigation of involvement of other viruses is described in section 1.4.2. A PCR strategy for the detection of known or as yet uncharacterised human herpesviruses in HD tumour tissue is discussed in chapter 5.

Studies of oncogene involvement in HD have largely been restricted to analyses of HD-derived cell lines (Jucker *et al.*, 1990). These studies have given variable results and the *in vivo* significance of these findings is unclear. No consistent chromosomal abnormalities have been detected in HD by cytogenetic studies (Sandberg, 1990) however, in recent years, a possible role for the t(14;18) translocation involving the IgH gene and the B-cell lymphoma-2 (*bcl*-2) gene has been suggested. The role of the *bcl*-2 gene is discussed in section 1.5. and our study investigating the presence of the t(14;18) translocation in HD is discussed in detail in Chapter 3.

Mutation of the p53 tumour suppressor gene is the most frequent abnormality detectable in human tumours. Studies of p53 expression in HD have detected p53 in the RS cells of 32-74% of cases (Doglioni *et al.*, 1991; Gupta *et al.*, 1992a; Niedobitek *et al.*, 1993). Utilising a single cell PCR analysis Trumper *et al.* (1993), detected a mutation of the p53 gene in five out of seven RS cells in one case examined. These studies suggest that p53 may play a role in the pathogenesis of a proportion of HD cases.

1.3. HERPESVIRUSES

To date over 100 herpesviruses have been partially or fully characterised of which 7 have been isolated from humans. Herpesviruses are widespread and at least one herpesvirus has been found in association with most species.

1.3.1. Family Herpesviridae

Inclusion in the family Herpesviridae is based on the architecture of the virion which includes (a) a core containing a linear double-stranded DNA (dsDNA) (Furlong *et al.*, 1972), (b) an icosahedral capsid, approximately 100-110 nm in diameter (Wildy & Watson, 1963), (c) the tegument (Morgan *et al.*, 1959, 1968) and (d) an envelope containing viral glycoproteins (Epstein, 1962). Herpesvirions have been reported to vary from 120-300 nm in size. DNA extracted from virions is linear and double-stranded ranging from 120-230 kilobases (kb) in length for the entire viral genome. Within the virion the DNA is linear however the genomes circularise immediately upon release from capsids into the nuclei of infected cells.

There are two distinct phases of the herpesvirus lifestyle known as lytic and latent phases. In the lytic cycle host cell DNA protein synthesis can be shut off leading to host cell death (Wagner & Roizman, 1969). The ability to establish latent infection, in which a limited number of viral genes are expressed, is characteristic of herpesviruses (Roizman, 1982). EBV latent gene expression is discussed in section 1.3.4.2.

1.3.2. Herpesvirus glycoproteins

The herpesvirus membrane glycoproteins play a major role in the early stages of viral infection and are important in determining the biological and pathogenic properties of the virus (reviewed by Spear, 1985). A number of glycoproteins of the alphaherpesvirus herpes simplex virus 1 (HSV-1) have been identified (gB, gC, gD, gE, gG, gH, gI, gJ, gK and gL) (Spear, 1985; Hutchinson *et al.*, 1992).

Glycoprotein B (gB) was the first glycoprotein shown to be required for virus infectivity (Sarmiento *et al.*, 1979). It is the most highly conserved glycoprotein between herpesviruses, with HSV-1 gB being the prototype (Spear, 1985). Chapter 5 describes the use of a PCR strategy to detect herpesvirus gB sequences in DNA from clinical samples utilising primers based on two well conserved regions of the gB and gB-like genes.

1.3.3. Classification of herpesviruses

The members of the family Herpesviridae have been sub-classified by the International Committee on the Taxonomy of Viruses into three subfamilies based on biological properties including host range, site of latent infection, cytopathology and length of replicative cycle (Roizman, 1992). These subfamilies are (a) alphaherpesvirinae, (b) betaherpesvirinae and (c) gammaherpesvirinae.

1.3.3.1. Alphaherpesvirinae

Members of the subfamily alphaherpesvirinae have a variable host range and relatively short reproductive cycle. They have been shown to spread rapidly in culture with efficient destruction of infected cells. In addition, they have the capability of establishing latent infection which is primarily localised to the sensory ganglia. Members of this subfamily include HSV-1, HSV-2 and varicella zoster virus (VZV).

1.3.3.2. Betaherpesvirinae

One characteristic of the subfamily betaherpesvirinae is the restricted host range. The reproductive cycle is long and infection in culture is slow. Infected cells often become enlarged (cytomegalia) and cultures carrying these viruses can be readily established. This subgroup also has the ability to establish latent infection and although this is generally observed in secretory glands and kidneys, other tissues may be infected. Human cytomegalovirus (HCMV) and the more recently identified human herpesviruses (HHV), HHV-6 & 7 have been assigned to this group.

1.3.3.3. Gammaherpesvirinae

All members of this subfamily have the ability to replicate in lymphoid cells *in vitro*. Lytic infection in epithelial and fibroblastic cells is also observed for some viruses of this group. Gammaherpesvirinae are specific for either T- or B-lymphocytes and infection is usually pre-lytic or lytic without production of infectious progeny. Latent virus infection is however frequently demonstrated in lymphoid tissue. Included in this group is the human herpesvirus EBV.

1.3.4. Epstein-Barr virus

EBV was originally identified in cultured lymphoblasts derived from Burkitt's lymphomas (BLs) of African patients (Epstein *et al.*, 1964). The EBV genome is a linear dsDNA of around 170 kb in length that circularises upon entry into the infected cell. Circularisation occurs by the joining of multiple homologous terminal repeat sequences of approximately 500 base pairs (bp) in length (Pritchett *et al.*, 1975; Kintner & Sugden, 1979).

EBV is a human B-lymphotropic herpesvirus (Jondal & Klein, 1973; Greaves *et al.*, 1975) which is carried in a persistent state by over 90% of the worlds population (reviewed by Evans & Niederman, 1989). Primary infection generally occurs in early childhood and is asymptomatic. Early studies suggested that EBV infected oropharyngeal cells in the first

instance (Sixbey *et al.*, 1984; Wolf *et al.*, 1984) and that the virus then spread from the epithelium to B-cells (Allday & Crawford, 1988). The exact site of persistence of the virus remains unclear, however recent reports suggest that B-cells are important in both primary infection and persistence of EBV (Niedobitek & Young, 1994). Late exposure and primary infection by the virus in adolescence or young adulthood results in the onset of a non-malignant lymphoproliferative disease, known as infectious mononucleosis (IM) or glandular fever, in approximately 50% of cases (Henle *et al.*, 1968). In addition to IM, a number of malignant disease processes have now been associated with the virus including BL (Zur Hausen *et al.*, 1970), B-cell lymphomas in immunocompromised individuals (Hamilton-Dutoit *et al.*, 1993) and undifferentiated nasopharyngeal carcinoma (UNPC) (Klein, 1979). These tumours exhibit different patterns of EBV gene expression as discussed in section 1.3.4.2. The precise role of EBV in these malignancies remains unclear. The association between EBV and HD is discussed in section 1.4.1.

1.3.4.1. EBV latent genes

Infection of human B-cells with EBV *in vitro* results in their immortalisation and the creation of a lymphoblastoid cell line (LCL) which is said to be latently infected (Pope *et al.*, 1968). The exact mechanism of immortalisation of B-cells remains unclear. A limited number of latent viral genes are expressed in LCLs and many studies have attempted to characterise their function (Kieff & Liebowitz, 1990). Of these genes, six encode nuclear proteins known as EBV nuclear antigens (EBNAs) -1, -2, 3A, -3B, -3C and leader protein (EBNA-LP). Three membrane proteins designated latent membrane protein (LMP) -1, -2A, -2B are also expressed. In addition two small non-translated RNA species termed the EBV-encoded RNAs (EBERs) have been identified. Expression of three of these viral proteins has been shown to be necessary for the immortalisation of B-cells (reviewed by Ring, 1994).

EBNA-1 is a DNA binding protein which is required for the episomal maintenance of the EBV genome (Yates *et al.*, 1984, 1985). The effect of EBNA-1 on malignant transformation remains unclear, however, expression of EBNA-1 in the B-cells of transgenic mice has been shown

to be associated with the development of lymphocytic lymphoma and leukaemia (Wilson & Levine, 1992).

Current evidence suggests that EBNA-2 and LMP-1 are essential in the immortalisation process of B-cells. EBNA-2 has been shown to increase the expression of LMP-1 (Abbot *et al.*, 1990) and the B-cell activation antigen CD23 (Wang *et al.*, 1990). The induction of CD23 appears to be a critical event in the immortalisation process and is detected at high levels in EBV immortalised B-lymphocytes (Kintner & Sugden, 1981).

LMP-1 has the ability to transform rodent fibroblasts *in vitro* and at present is the only EBV latent gene product capable of transformation in isolation (Wang *et al.*, 1985). LMP-1 either singularly or in combination with EBNA-2 has been shown to upregulate CD23 (Wang *et al.*, 1987, 1990). In addition LMP-1 has the ability to upregulate the *bcl-2* gene as discussed in section 1.2.5.

1.3.4.2. Patterns of EBV latent gene expression

In B-cells three patterns of expression of the EBV latent proteins have been described, designated Lat I, Lat II, and Lat III. In Lat I only the EBNA-1 protein is expressed. In Lat II, both EBNA-1 and the LMPs are expressed and in Lat III all of the EBV latent gene products are present (Rowe *et al.*, 1992). Various malignancies have been associated with different patterns of EBV gene expression. Lat I expression is seen in BL (Rowe *et al.*, 1986) whereas Lat III latency is observed in lymphomas in immunosuppressed persons and is similar to that observed in LCLs (Young *et al.*, 1989). In UNPC an intermediate pattern, or Lat II is described (Young *et al.*, 1988).

1.3.4.3. EBV subtypes

Two strains of EBV have been described which differ in biological properties and can be distinguished immunologically and molecularly on the basis of differences in the EBNA-2 and EBNA-3 genes (Zimber *et al.*, 1986; Young *et al.*, 1987; Sample *et al.*, 1990). EBV type A (or type 1)

has been shown to transform cell lines more readily than type B (or type 2) *in vitro* (Rickinson *et al.*, 1987). Geographical differences between the two subtypes have indicated that EBV type 1 is the predominant subtype found in Western countries whereas type 2 is restricted to Papua New Guinea and Africa (Young *et al.*, 1987). Recent evidence, based on PCR subtyping suggests that type 2 virus may be more widely distributed in the West (Sixbey *et al.*, 1989).

1.4. VIRAL INVOLVEMENT IN HD

1.4.1. EBV in HD

The first association between EBV and HD was made on the basis of seroepidemiological studies which showed elevated antibody titres to EBV in cases compared to controls (Henle & Henle, 1973; Levine *et al.*, 1971). Patients with a past history of IM, which is a disease associated with late exposure to the EBV, have a three-fold increased risk of developing HD (Pagano *et al.*, 1973; Munoz *et al.*, 1978). Early studies failed to detect EBV genomes in HD tumour tissue (Lindahl *et al.*, 1974) however in later years EBV genomes were demonstrated in 4 of 21 cases of HD utilising Southern blot analysis (Weiss *et al.*, 1987a). The sensitivity of detection of EBV in this study was enhanced by the use of cloned EBV probes, in particular a probe to the major internal repeat sequence of the virus (Arrand *et al.*, 1981). Using similar methodology to that described above a number of laboratories have detected EBV in a proportion (up to 41%) of HD cases (Anagnostopoulos *et al.*, 1989; Boiocchi *et al.*, 1989; Gledhill *et al.*, 1991; Jarrett *et al.*, 1991).

The clonality of an EBV-infected cell population can be determined by analysis of the terminal repeat sequences of the EBV genomes using Southern blot analysis (Raab-Traub & Flynn, 1986). Using this methodology it has been shown that the EBV-infected cells in HD are clonal (Weiss *et al.*, 1989; Gledhill *et al.*, 1991). In more recent years the question of whether the EBV genomes are present in the RS cells or in the reactive infiltrate has been answered using probes directed at the EBV EBER RNAs. The presence of these abundantly transcribed RNAs has enabled the development of a sensitive RNA *in situ* hybridisation

assay which can be performed on paraffin-embedded tissue (Howe & Steitz, 1986). The first study applying this technique in HD showed that EBV was present within the RS cell population in six of eight cases positive for EBV on Southern blot analysis (Wu *et al.*, 1990). In a later study, RS cells from 16 of 17 cases which were EBV-positive on Southern blot analysis and shown to be clonal by this method were EBER-positive (Armstrong *et al.*, 1992b). These data suggest that, in a proportion of HD cases, EBV within the RS cells represents a clonal expansion of a single EBV-infected cell. These findings have led us to suggest that such cases should be designated EBV-associated (Armstrong *et al.*, 1992b).

A number of studies have shown that LMP-1 is detectable within RS cells in HD lesions (Pallesen *et al.*, 1991; Murray *et al.*, 1992; Armstrong *et al.*, 1992a). This protein is highly expressed within the RS cells in the majority of cases shown to be EBV-positive by EBER *in situ* techniques (Armstrong *et al.*, 1992b). The high level of LMP-1 expression by RS cells suggests that EBV is playing a role in the pathogenesis of the disease process. Indeed LMP-1 is known to upregulate a number of molecules including CD23, cellular adhesion molecules and the *bcl*-2 proto-oncogene (section 1.3.4.1.). CD23 is expressed in the RS cells in a minority of cases but no clear association has been found between the expression of LMP-1 and CD23 in HD (Herbst *et al.*, 1991; Armstrong *et al.*, 1992b). We have further shown that the expression of *bcl*-2 does not correlate with the presence of EBV LMP-1 (Armstrong *et al.*, 1992a). The exact role played by LMP-1 in the pathogenesis of HD remains unclear. There is no evidence that EBNA-2 is expressed by RS cells however a recent study has confirmed the supposition that EBNA-1 is expressed (Grasser *et al.*, 1994). The pattern of EBV genome expression in HD therefore conforms to that designated Lat II (section 1.3.4.2.), a pattern similar to that observed in UNPC.

1.4.1.1. EBV association with age in HD

It is clear that EBV is present in the RS cells of only a proportion of cases. Southern blot studies have reported an excess of EBV-positive cases in the MC subtype of HD in contrast to that seen in the NS

subtype (Weiss *et al.*, 1987a; Staal *et al.*, 1989; Gledhill *et al.*, 1991). Most large studies using immunohistochemical or *in situ* techniques to determine EBV status have found a significant association between EBV positivity and MCHD as compared to NSHD (Pallesen *et al.*, 1991; Murray *et al.*, 1992; Armstrong *et al.*, 1994). The number of cases of LPHD and LDHD studied have generally been insufficient to accumulate statistically significant data, however, most LPHD cases have been EBV-negative (Weiss *et al.*, 1991; Pallesen *et al.*, 1991). Analysis of the relationship between EBV-positivity and age has revealed striking results (Jarrett *et al.*, 1991). In the latter study we demonstrated that EBV genomes were detectable in tumour samples from most of the paediatric and older cases of HD examined. In contrast, within the young adult group EBV genomes were demonstrable in <15% of cases. This supports the "two disease" hypothesis proposed for HD (Gutensohn, 1982; Alexander *et al.*, 1991a). The low level of EBV-positivity within the young adult group, particularly those of NS subtype, would suggest that other factors are involved in these cases (Jarrett *et al.*, 1991).

More recently a comparison of EBV-association in paediatric cases of HD in developed and developing countries was made utilising the EBER *in situ* hybridisation technique (Armstrong *et al.*, 1993). The results obtained confirmed previous findings that EBV was present in the majority of cases of childhood HD and, in particular, those under 10 years of age. No significant differences were noted on comparison of geographical locale and different ethnic or socioeconomic backgrounds. The results of this study and those described above suggest that EBV-associated cases in HD fall into defined epidemiological groups. In the context of EBV-positivity these results do not provide support for the polio model but are consistent with the multiple aetiology hypothesis as discussed in section 1.2.5.

1.4.2. Other viruses in HD

EBV is rarely associated with young adult cases of HD, in particular the NS subtype, as described. It is in this group that the strongest evidence exists for an infectious aetiology. The delayed exposure hypothesis, as discussed in section 1.2.5., suggests that the infectious agent may be a

common childhood pathogen. Possible candidates are herpesviruses other than the EBV. At present serological studies have not shown an association between HD and the herpesviruses HSV, VZV and CMV (Henderson *et al.*, 1973; Langenhuysen *et al.*, 1974; Catalano & Goldman, 1972; Evans & Gutensohn, 1984). In contrast to these studies, several groups have reported elevated antibody titres to the herpesvirus HHV-6 in HD (Ablashi *et al.*, 1988; Biberfeld *et al.*, 1988; Clark *et al.*, 1990). In one study raised antibody levels to the virus were found to be most pronounced in young adult patients (Clark *et al.*, 1990). These studies suggested that HHV-6 may be aetiologically involved, however, HHV-6 DNA sequences were not detected in any of 47 cases of HD using Southern blot analysis (Gledhill *et al.*, 1991). In a later study HHV-6 sequences were detected in 3 of 25 cases examined by Southern blot analysis or PCR and all three cases were young adult women with NSHD (Torelli *et al.*, 1991). These results are tantalising although HHV-6 genomes have not as yet been localised to the RS cells within HD tumour tissue. HHV-6 may play a role in a small proportion of cases however the low rate of genome positivity would suggest that HHV-6 is unlikely to account for the young adult peak. Another herpesvirus may be involved in these cases. Chapter 5 describes a PCR technique for the detection of herpesvirus sequences in DNA from HD tumour material.

1.5. BCL-2

One of the most prevalent chromosomal translocations found in human lymphoma is the t(14;18) (q32;q21) and the location of the IgH gene on chromosome 14q32 provided a means to clone this juncture. An aberrant IgH gene rearrangement proved to be the chromosomal breakpoint and the subsequent analysis revealed a candidate proto-oncogene *bcl*-2 at position 18q21 (Cleary & Sklar, 1985; Bakhshi *et al.*, 1985; Tsujimoto *et al.*, 1985). The translocation is associated with >75% of follicular lymphomas and 30% of diffuse lymphomas (Yunis *et al.*, 1987; Weiss *et al.*, 1987b). Approximately 60% of the translocations occur within a specific region designated the major breakpoint region or mbr (Cleary & Sklar, 1985; Bakhshi *et al.*, 1987).

As described above the translocation juxtaposes the *bcl*-2 gene situated on chromosome 18 with the IgH gene on chromosome 14. This causes dysregulation of the *bcl*-2 gene and inappropriate levels of Bcl-2 are expressed (Graninger *et al.*, 1987; Seto *et al.*, 1988). A number of studies have shown that abundant *bcl*-2 mRNAs are expressed in all t(14;18) carrying cell lines (Tsujimoto *et al.*, 1985; Cleary *et al.*, 1986; Chen-Levy, 1989). Transgenic mice bearing a *bcl*-2/Ig mini gene were found to exhibit a polyclonal expansion of resting B-cells which displayed prolonged cell survival but no increase in cell cycling (McDonnell *et al.*, 1989, 1990). In addition, dysregulated Bcl-2 protein expression has been shown to extend the survival of certain haematopoeitic cells lines following growth factor deprivation (Vaux *et al.*, 1988; Nunez *et al.*, 1990).

The expression of Bcl-2 is not restricted to cells carrying the t(14;18) translocation (Pezella *et al.*, 1990) and Bcl-2 is highly expressed in normal pre-B cells but down-regulated in mature B-cell lines. It has now been demonstrated that Bcl-2 is a membrane protein and that overexpression of this protein blocks programmed cell death and thereby increases cell survival (Hockenbery *et al.*, 1990). The subcellular location of Bcl-2 is controversial, however Bcl-2 protein in overexpressing cells has been shown to be associated with the nuclear envelope and endoplasmic reticulum as well as with the mitochondria (Jacobson *et al.*, 1993).

Activation of EBV latent gene products has been shown to protect human B-cells from apoptosis (Gregory *et al.*, 1991). Transfection experiments have confirmed that this protection from apoptosis is conferred through the expression of a single latent gene product, LMP-1. These experiments showed that LMP-1 mediated this effect by upregulating the expression of Bcl-2 (Henderson *et al.*, 1991). Chapter 3 discusses the investigation of the t(14;18) translocation in HD and correlates results with both Bcl-2 and LMP-1 expression.

1.6. SCID MICE

CB-17 severe combined immunodeficient (SCID) mice are characterised by a homozygous mutation of the SCID locus in the mouse which disrupts both B-and T-cell lymphoid development. This results in low serum Ig levels and the lack of functional T-cells (Bosma *et al.*, 1983). The defect has been mapped to chromosome 16 in the mouse (Bosma & Carroll, 1991; Miller *et al.*, 1993). The lack of functional B-and T-cells is due to a defect in V(D)J recombination (reviewed by Bosma & Carroll, 1991). In addition SCID mice have a defect in DNA double-stranded break-related repair (Hendrickson *et al.*, 1991).

Although SCID mice have a severe immune deficit the overall ability of these mice to support lymphoid development is not impaired. The deficiency of mature B-and T-cells can be corrected by engrafting adult SCID mice with bone marrow or fetal liver cells from normal mice (Bosma *et al.*, 1983; Custer *et al.*, 1985). These initial observations prompted other investigators to attempt the xenogeneic engraftment of human lymphoid cells into SCID mice (Mosier *et al.*, 1988; McCune *et al.*, 1988). The success of these experiments and others in more recent years (Lubin *et al.*, 1991; Lapidot *et al.*, 1992) have encouraged the use of the SCID mouse as a model for the investigation of human disease.

Many different diseases have now been investigated using the SCID mouse model (reviewed by Hendrickson, 1993) including multiple myeloma (Bellamy *et al.*, 1993), leukaemia (Cesano *et al.*, 1993; Kamel-Reid *et al.*, 1989; Lapidot *et al.*, 1994) and human lymphoma (Garnier *et al.*, 1993; Boyle *et al.*, 1992). In addition SCID mice have been used to support the *in vivo* growth of human lymphoma cell lines (Chang *et al.*, 1992) and lymphomas from immunosuppressed post-transplantation patients (Waller *et al.*, 1993). The study of viral infections in this model has also been reported in the literature e.g., human cytomegalovirus (HCMV) (Mocarski *et al.*, 1993). EBV-induced human B-cell lymphoproliferations have been more extensively studied. Between 30% and 80% of SCID mice develop human B-cell lymphoproliferations following transplantation of lymphocytes from EBV-seropositive donors (Mosier *et al.*, 1992; Nakamine *et al.*, 1991; Rowe *et al.*, 1991).

Recently attempts have been made to utilise the SCID mouse model for the study and investigation of HD. Kapp *et al.*, (1992) showed that HD-derived cell lines which could not be propagated in nude mice grew in SCID mice. In this study tumour growth was also observed in SCID mice following injection of primary HD tumour material. In another study successful transplantation of a HD-derived cell line (HD-MyZ) into SCID mice gave rise to a large tumour mass with the presence of RS-like cells and a phenotype similar to that of the parental *in vitro* cell line (Bargou *et al.*, 1993).

The only previously reported study of transplantation of HD into SCID mice described 3/13 cases in which B cell lymphoma developed following transplantation of HD tumour fragments (Kapp *et al.*, 1993). The aforementioned studies would suggest that the SCID mouse model may be a useful tool for the *in vivo* study of HD and perhaps aid the further characterisation of the malignancy. These implications are discussed in further detail in chapter 4, which describes our attempt to engraft tumour material derived from HD and NHL tumour biopsies into SCID mice.

1.7. AIMS OF PROJECT

The aims of this study were to investigate the involvement of oncogenes and viruses in the pathogenesis of HD. The presence of the t(14;18) translocation was investigated and the results correlated with Bcl-2 and EBV LMP-1 expression. In order to study the role of other oncogenes and viruses we explored the use of the SCID mouse model as a means to propagate HD tumour material. In addition a new strategy was devised for the search for herpesvirus sequences in HD. This will be used to determine whether a herpesvirus other than EBV is present in the non EBV-associated cases of the disease, in particular, young adult NS cases.

CHAPTER 2
GENERAL MATERIALS AND METHODS

2.1. MATERIALS

The following chapter describes the materials and methods frequently used in the work relating to this thesis. The sources of commonly used materials are listed in Table II.I. and the composition of routinely used stock solutions and buffers in Table II.II. Additional materials and chemicals not listed are specified elsewhere in the text. All chemicals used were of Analar™ or Molecular Biology grade and unless otherwise stated were obtained from The Sigma Chemical Company Limited (Poole, Dorset, UK) or BDH (Poole, Dorset, UK). All restriction and DNA modifying enzymes were obtained from Life Technologies (Gibco/BRL, Paisley, UK). Ultra pure de-ionised water obtained from a reverse osmosis filtration system (Millipore) was used in all enzymatic manipulations and for the re-suspension of DNA. De-ionised water obtained from a MilliQ water filtration system (Millipore) was used in all other procedures. S.I. units are used throughout this thesis.

2.2. MANIPULATION OF HIGH MOLECULAR WEIGHT DNA

2.2.1. DNA extraction from frozen tissue

Frozen tissue was disrupted using a Stomacher 80 (Colworth) after the addition of 5-25 mL of extraction buffer (Table II.II.). The volume of buffer added was dependent on tissue mass. Proteinase K was added to a final concentration of 100 μg/mL and sodium dodecyl sulphate (SDS) to a concentration of 0.5%. The cell suspension was digested for 1 hour at 50°C or overnight at 37°C.

In order to remove protein from the tissue sample, an equal volume of phenol equilibrated with Tris pH 8.0 (Rathburn Chemical Company, Walkerburn, UK) was added at room temperature and the tube was then slowly inverted, to allow aqueous and organic phases to mix completely. After centrifugation at 2000 rpm for 10 minutes (Denley BR401 centrifuge) to separate the aqueous and organic phases, the upper aqueous phase was transferred to a fresh tube. To prevent shearing of the high molecular weight (HMW) DNA, transfer was carried out using a wide bore polypropylene pastette (Alpha Labs). This procedure was

Table II.I. Sources of routinely used materials

Materials	Source
Apparatus for agarose gel electrophoresis	BRL
Apparatus for polyacrylamide gel electrophoresis	Biorad
Corex centrifuge tubes (30 mL)	Sarstedt
Falcon tubes (15 mL, 50 mL)	A & J Beveridge Ltd
Filter paper (Whatman)	Fisons
1.5 mL screw cap tubes	Elkay
Nylon membrane (Hybond-N)	Amersham
Oakridge centrifuge tubes (30 mL)	Sarstedt
Petri dishes	Fisons
Pipette tips	Greiner
Polaroid film (Type 57)	Genetic Research Instrumentation

Table II.II. Stock solutions and buffers

8% acrylamide solution	acrylamide stock solution (30%)	260 mL/L
	N,N methylene bisacrylamide (2%)	130 mL/L
	TBE	1 x
Alkali buffer	sodium hydroxide	0.5M
	sodium chloride	1.5M
	adjusted to pH 12.0	
Denhardt's solution (100 x)	Ficoll	2%
	Bovine serum albumin (Fraction V)	2%
	polyvinylpyrolidine	2%
Elution buffer (DEAE membrane)	sodium chloride	1M
	Tris pH 9.0	50mM
	EDTA	1mM
DNA extraction buffer	sodium chloride	144mM
	Tris pH 8.0	10mM
	EDTA pH 8.0	1mM
10 x gel loading buffer	bromophenol blue	0.42%
	xylene cyanol	0.42%
	glycerol	50%
Neutralising buffer	Tris base	0.5M
	sodium chloride	3M
	concentrated hydrochloric acid	3.3%
	adjusted to pH 8.0	

Table II.II. Stock solutions and buffers (continued)

Potassium acetate (3M/5M)	potassium acetate stock solution (5M)	60 mL
	glacial acetic acid	11 mL
	deionised water to final volume	100 mL
SSC (1X)	sodium chloride	0.5M
	sodium citrate	0.015M
Southern hybridisation buffer	Formamide	50%
	sodium dodecyl sulphate	0.1%
	Denhardt's solution	5x
	Tris pH 7.4	50mM
	EDTA pH 8.0	10mM
TAE	Tris pH 8.0	40mM
	sodium acetate	20mM
	sodium chloride	20mM
	EDTA pH 8.0	2mM
TBE	Tris pH 8.0	90mM
	boric acid	90mM
	EDTA pH 8.0	2.25mM
TE	Tris pH 8.0	10mM
	EDTA pH 8.0	1mM
TNE	Tris	10mM
	sodium chloride	0.1M
	EDTA pH 8.0	1mM

Table II.III. Composition of bacterial media

Luria-Bertani (LB) medium	sodium chloride	170mM
	bactotryptone	1%
	bacto-yeast extract	0.5%
	adjusted to pH 7.0	
L-Agar	as LB medium with agar	1.5%
SOC	bactotryptone	2%
	yeast extract	0.5%
	sodium chloride	10mM
	potassium chloride	2.5mM
	magnesium chloride	10mM
	magnesium sulphate	10mM
	glucose	20mM
	adjusted to pH 7.0	

was performed twice and then repeated using chloroform as the organic solvent.

The DNA was precipitated by pipetting the aqueous phase into 98% ethanol and whirling gently. Using a sealed glass pasteur pipette, the DNA precipitate was spooled from the ethanol. If the DNA appeared degraded, or if there were insufficient DNA to visualise the precipitate, the ethanol was placed in a 30 mL Corex tube and the DNA pelleted by spinning at 10,000 rpm for 30 minutes in a JA-20 rotor (Beckman Instruments Ltd, High Wycombe, UK). One millilitre of 70% ethanol was added to wash the pellet and to remove excess salt, followed by a final wash with 1 mL of 98% ethanol. The DNA was air dried at room temperature and resuspended in TE buffer (Table II.II.).

In order to assess the concentration of DNA, optical density (OD) readings at wavelengths of 260nm and 280nm were obtained using a spectrophotometer (Pharmacia Ltd, Milton Keynes, UK). The concentration of DNA was calculated on the basis that 50 μg/mL of dsDNA has an OD_{260nm} of 1.0. The ratio of the OD_{260nm} to the OD_{280nm} reading was obtained to assess the purity of the DNA sample.

2.2.2. Restriction endonuclease digestion of HMW DNA

Ten micrograms of HMW DNA were digested in a 50 μL volume containing 1 x reaction buffer (Gibco/BRL, Paisley, UK) and spermidine at a final concentration of 3 mM. Five units of the appropriate restriction endonuclease were added per μg of DNA and the reactions were incubated at 37°C for 16-20 hours, to allow digestion to take place. Enzymatic reactions were stopped by the addition of EDTA (pH 8.0) to a final concentration of 10 mM, when digested samples would not be analysed immediately.

2.2.3. Agarose gel electrophoresis

Endonuclease-digested HMW DNA was electrophoresed on a 0.8% agarose gel unless otherwise stated. The agarose was dissolved by

boiling in 1 x TBE buffer (Table II.II.) and the gel mix was then poured into a Perspex tray and allowed to set at room temperature with a well-forming comb in place. The gel was completely submerged in 1 x TBE in an electrophoresis tank, before carefully removing the comb. Gel loading dye was added to the digested DNA to a 1 x concentration and the gel was electrophoresed for 16-20 hours at a constant 32 V. A molecular weight standard, Lambda Hind III marker, was run alongside the DNA sample to allow sizing of digested fragments. The DNA was stained by immersing the gel in a 0.5 μg/mL aqueous solution of ethidium bromide for 25 minutes then destained by immersing in de-ionised water for 20 minutes. The DNA was visualised on a transilluminator using short wave UV light (Foto/Prep I, Fotodyne Inc.) and photographed with a Polaroid MP40 Land camera using high speed Polaroid Type 57 film (Genetic Research Instrumentation Ltd, Dunmow, UK).

2.2.4. Southern blotting

Agarose gels were blotted by capillary action assisted by gravity. The gels were placed in alkali buffer for 40 minutes to denature the DNA followed by a further 40 minutes incubation in neutralisation solution. Prior to blotting, the gels were placed in 10 x SSC solution (transfer buffer) for 10 minutes. A piece of nylon membrane (Hybond-N, Amersham International plc, Little Chalfont, UK) was cut to gel size and pre-soaked in transfer buffer. DNA was transferred in a downward direction by placing the nylon membrane and gel on a layer of absorbent material and overlaying the gel with a sponge saturated in transfer buffer. To prevent evaporation the blot was sealed in plastic wrapping and left for 16-20 hours to allow transfer to take place. In order to remove extraneous gel material, the nylon membrane was washed briefly in a solution of 3 x SSC. DNA was irreversibly bound to the membrane by baking in an oven at 80^{o}C or by cross-linking with a UV light source at 120 joules/cm^{2} for 0.8 minutes (UV Stratalinker 1800, Stratagene).

2.3. AMPLIFICATION AND PREPARATION OF PLASMID DNA

2.3.1. Transformation of competent bacteria

Competent bacteria, unless otherwise stated, were *E. coli* strain HB101 obtained from Gibco/BRL. Bacterial cells were thawed on ice and a 100 μL aliquot placed in a 2059 Falcon tube (A & J Beveridge). Between 10 and 100 ng of the appropriate plasmid DNA were added to the cells and placed on ice for 30 minutes. The mixture was heat shocked at 42°C for 45 seconds without agitation and placed immediately on ice for 2-3 minutes. Nine hundred microlitres of L-broth or SOC (Table II.III.) were added and the reaction placed in a shaking incubator for 1 hour. Bacteria were spread onto L-agar plates (Table II.III.) containing the appropriate antibiotic and placed at 37°C overnight.

2.3.2. Growth of bacteria and amplification of plasmid DNA

Following transformation, a single colony was picked, using a sterile loop, into 5 mL of L-broth containing the appropriate antibiotic and placed at 37°C with constant agitation overnight. The 5 mL culture was poured into a flask containing 500 mL of L-broth plus antibiotic and subsequently incubated overnight in an orbital incubator at 37°C.

2.3.3. Bacterial freezes

Bacterial freezes were routinely prepared from plasmids which had not previously been transformed into competent bacteria within this department. A 5 mL overnight culture was prepared from the appropriate plasmid-containing bacteria. One millilitre of the culture was added to 0.5 mL of 80% glycerol in water and was quickly frozen by placing in a beaker containing dry ice and 98% ethanol. The bacterial freeze was stored at -80°C.

2.3.4. Preparation of plasmid DNA

Following step 2.3.2., cells were harvested by centrifugation at 7000 rpm for 10 minutes (JS-7.5 rotor, Beckman). The supernatant was discarded and the cells washed in 100 mL of TNE buffer followed by a further centrifugation. Residual supernatant was removed and the pellet was resuspended in 4 mL of buffer containing 50 mM glucose, 10 mM EDTA, 25 mM Tris.HCl and 0.5% lysozyme. After a 5 minute incubation at room temperature, 10 mL of an alkali solution containing 0.2N NaOH and 1% SDS were added to the bacterial suspension and mixed thoroughly. The preparation was placed on ice for 10 minutes, before the addition of 12 mL of potassium acetate solution (Table II.II.) and incubation on ice for a further 10 minutes. The lysed bacteria were pelleted by centrifugation at 19,000 rpm for 30 minutes in a JA-20 rotor (Beckman). The supernatant was decanted into two glass Corex tubes and 0.6 volumes of isopropyl alcohol were added and mixed to allow the precipitation of the plasmid DNA. The mix was spun for 30 minutes at 10,000 rpm in a JA-20 rotor and the supernatant removed. Five millilitres of 70% ethanol were added to the DNA pellet to remove salt followed by an additional spin at 10,000 rpm for 10 minutes. The pellet was left to drain briefly before resuspending in 5 mL of TE buffer. The plasmid DNA was purified on an ethidium bromide/caesium chloride gradient using methodology previously described by Maniatis *et al.*, (1982), and precipitated. Mini-plasmid preparations were performed on a smaller scale essentially as described above but without the addition of lysozyme in the initial resuspension step. The concentration of DNA was assessed spectrophotometrically as described in section 2.2.1. or by comparison of ethidium bromide staining intensity to that of known concentrations of DNA following agarose gel electrophoresis.

2.3.5. Digestion of plasmid DNA

Plasmid digestions were carried out using similar methodology to that described in section 2.2.2. but with 1 μg of DNA as starting material and without the addition of spermidine. A two hour incubation at 37°C was sufficient for digestion to take place. In order to check the authenticity of the plasmid, the digest was run on a 0.8% agarose gel adjacent to 1 μg

of Lambda Hind III DNA marker (Gibco/BRL). The gel was stained and photographed as described in section 2.2.3.

2.3.6. Preparation of plasmid DNA insert for use as probe

Twenty five micrograms of the required plasmid were digested in a 200 μL volume containing 1 x appropriate buffer (as supplied by manufacturer) and 3-5 units of restriction enzyme per μg of DNA, essentially as described in section 2.2.2. In order to check if digestion were satisfactory, 1% of the digestion mix was electrophoresed on an agarose gel. If satisfactory the remaining digestion was run on a 0.8% agarose gel containing 0.5 μg/mL of ethidium bromide for 2-3 hours at a constant 40 V or overnight at 15 V.

Using a scalpel blade a small incision was made in the gel directly below the DNA fragment of interest. A piece of DEAE membrane (Schleicher and Schuell, West Germany) was cut to size and placed in the gel space. The gel was electrophoresed until the DNA fragment of interest had migrated onto the membrane. The membrane was removed and placed in sufficient elution buffer (Table II.II.) to cover the membrane, and incubated at 65°C for 1 hour. To ensure that complete elution had taken place the membrane was examined for the presence of residual ethidium bromide-stained DNA using a hand held short wave UV light source (model UVGL-58, UVP inc, USA). A phenol/chloroform extraction was performed as described in section 2.2.1. and after addition of NaCl to a final concentration of 0.4 M the DNA was precipitated in 2 volumes of ethanol at -20°C for 1 hour. DNA was pelleted by centrifugation at 13000 rpm in a microcentrifuge (MSE) for 30 minutes. The pellet was washed briefly with a solution of 70% ethanol to remove extraneous salt and again pelleted as described above. The DNA pellet was briefly dried and resuspended in TE buffer or de-ionised water.

2.4. THE USE OF RADIOACTIVELY-LABELLED PROBES

^{32}P-radiolabelled probes were used for the specific detection and investigation of membrane-bound DNA target sequences.

2.4.1. Labelling of DNA plasmid probes by random priming

The preparation of plasmid DNA for use as probe is described in section 2.3.6. Plasmid insert or linearised plasmid were labelled by random priming using a commercially available kit (Amersham International). Fifty nanograms of appropriate DNA in a 25 μL volume were denatured by boiling for 5 minutes and placed immediately on ice to cool. Ten microlitres of buffer containing the nucleotides dATP, dGTP and dTTP in Tris-HCl, pH 7.8 with magnesium chloride and 2-mercaptoethanol were added to the plasmid solution. Five microlitres of bovine serum albumin (BSA) containing random hexanucleotides were then added to the mix and this was pulsed briefly in a benchtop centrifuge (MSE microcentrifuge, Fisons Loughborough, UK). The DNA was labelled by the addition of 3 MBq of ^{32}P-labelled alpha dCTP (specific activity 800 Ci/mM) and 2 units of DNA polymerase I (Klenow fragment). The labelling reaction was placed at room temperature for 4-15 hours.

In later experiments a radiolabelled nucleotide solution containing a coloured dye (Redivue, Amersham International) was utilised for ease of handling. Three Megabequerels of the ^{32}P-labelled nucleotide (specific activity 3000 Ci/mM) were added and the reaction incubated for 30 minutes at 37^{o}C. Unincorporated nucleotides were removed from the reaction by Sephadex G50 (Pharmacia LKB) gel column filtration. Sephadex columns were either poured manually or commercial NICK columns (Pharmacia LKB) were used following the manufacturers instructions.

2.4.2. Hybridisation with plasmid probes

Prior to hybridisation nylon filters containing bound DNA were soaked briefly in a solution of 3 x SSC, 0.1% SDS and placed in a 50 mL Falcon

tube (Table II.I.) with the bound DNA on the inside of the tube. Two point five millilitres of the hybridisation buffer (Table II.II.) were added to the tube. The filters were pre-hybridised for a minimum of 4 hours with continuous rotation at 37°C to eliminate non-specific binding of probe. The hybridisation conditions were calculated utilising a formula similar to McConaughy *et al.*, (1969) where T_m = 81.5+16.6log M+0.41(G+C%)-500/n-0.61(%formamide) where M is the salt concentration and n the average probe size in bp. For the purposes of this calculation M=0.45 and n=100. Hybridisations were routinely performed at 25°C below the calculated T_m of the DNA duplex in 50% formamide buffer. Following the addition of probe at a concentration of 10^6 counts per minute (cpm) per mL of buffer, hybridisation was performed with continuous rotation for 16-20 hours at 37°C.

Filters were rinsed twice at room temperature in 2 x SSC, 0.1% SDS for 10 minutes to remove excess probe. Further stringent washes were carried out at conditions appropriate for the permitted degree of mismatch between probe and target sequence. Routinely, two washes were performed at 65°C in 0.5 x SSC, 0.1%SDS for 40 minutes. After removing excess moisture, the filters were wrapped in Saran wrap (Scotlab Bioscience Ltd, Coatbridge, UK) and placed with photographic film (Hyperfilm, Amersham International) in an autoradiographic cassette containing an intensifying screen. The cassette was placed at -20°C for 1-14 days before developing the film.

2.4.3. 5'-end labelling of oligonucleotides

Oligonucleotides, for use as probes, were obtained as described in section 2.5.1. Five prime end-labelling reactions were performed in a 100 μL volume containing 70 pmoles of oligonucleotide, 1 x kinase buffer (50 mM Tris pH7.5, 10 mM magnesium chloride, 5 mM DTT, 1 mM spermidine, 1 mM EDTA pH 8.0), 2 μL of T4 Polynucleotide kinase and 70 pmoles of ^{32}P γATP (3000 Ci/mM). Following incubation of the reaction for 1 hour at 37°C, unincorporated nucleotides were removed as described in section 2.4.1.

2.4.4. Hybridisation with oligonucleotide probes

Following PCR amplification (section 2.5.2.) products were transferred to nylon membrane by electroblotting (section 2.5.7.) and radioactively-labelled oligonucleotides were used for the detection of specific PCR products. Hybridisation was performed using similar methodology to that described in section 2.4.2. at 37°C with continuous rotation. After empirical testing, probes of 20-30 bp in length were hybridised in 25% formamide buffer (Table II.II.) containing 6 x SSC. Probes 30-35 bp in length were hybridised in 35% formamide buffer containing 6 x SSC. One million cpm of probe were added per mL of hybridisation mix and the reaction allowed to proceed at 37°C for 16-20 hours.

Filters were rinsed twice in a solution of 6 x SSC, 0.1% SDS for 10 minutes at room temperature, to remove excess probe. This was followed by two stringent washes at 60°C for 30 minutes in the wash solution described above. Filters were then briefly dried and exposed to film as discussed previously (section 2.4.2.).

2.5. THE POLYMERASE CHAIN REACTION

The specific PCR amplification of a target DNA sequence was achieved using methodology essentially as described by Saiki *et al.*, (1985). The procedures used and the optimisation of standard PCR techniques employed in this thesis are described below. Specific conditions and novel PCR techniques are detailed elsewhere in the text.

2.5.1. Oligonucleotide synthesis

Oligonucleotides for use as primers were manufactured on an automated synthesiser and cartridge purified unless otherwise stated (Alta Bioscience, University of Birmingham, UK). The primers were resuspended in ultra pure water and the concentration assessed as described in section 2.2.1. assuming that 30 μg/mL of oligonucleotide has an OD_{260nm} of 1.0.

Oligonucleotides for use as PCR probes were manufactured on an automated synthesiser Model 381A (Applied Biosystems) within the Department of Veterinary Pathology. The oligonucleotides were cleaved from their cartridge by flushing with 2 mL of 30% ammonia solution over a 2 hour period. De-protection of the oligonucleotide was achieved by incubating in the ammonia solution at 55°C overnight. The oligonucleotide was dried by centrifugation under vacuum (Savant Speedvac SC100) and resuspended in water. The concentration of the DNA solution was assessed as described above.

2.5.2. Standard PCR reaction

Standard PCR reactions were performed in a 50 μL volume containing target DNA, 200 μM of each deoxynucleoside triphosphate (dATP, dGTP, dCTP, dUTP) (Cambio, Cambridge, UK), 1.5 mM magnesium chloride buffer containing 50 mM potassium chloride, 10 mM Tris pH8.2, 0.01% gelatin, primers at the established optimal concentration (section 2.5.3.) and 1 unit of Amplitaq DNA polymerase (Perkin-Elmer). To prevent evaporation of the reaction mix during thermal cycling the reaction was overlaid with 2 drops of mineral oil.

The reaction mix was heated to 95°C for 7 minutes to denature the DNA in a thermal cycling machine (Thermocycler, Perkin-Elmer) and forty cycles of amplification performed. In each cycle samples were ramped to a temperature of 94°C over 2 minutes and held at this temperature for 10 seconds. The temperature was then reduced to 55°C over 2 minutes and the primers annealed for a 10 second period. To allow extension of the primers the temperature was gradually increased to 72°C over a 1 minute period and held at this temperature for 30 seconds. A final primer extension time of 7 minutes at 72°C was performed following completion of the thermal cycles. The reaction was then cooled to 6°C.

2.5.3. Optimisation of primer concentration

Optimisation of primer concentration was achieved by titrating the primers from a concentration of 4 μM to 0.125 μM using doubling

dilutions. The reaction was performed in a 50 μL volume using an appropriate amount of positive control DNA as template. All other constituents and cycling conditions were as stated in section 2.5.2. The optimal concentration was assessed by visualisation of the PCR products following electrophoresis and ethidium bromide-staining of polyacrylamide gels (section 2.5.6.).

2.5.4. Optimisation of $MgCl_2$ concentration

Optimal $MgCl_2$ concentrations for individual primer pairs were assessed by varying the $MgCl_2$ concentration from 3.0 mM to 0.5 mM in grades of 0.5 mM. The PCR was performed as standard (section 2.5.2) and analysed as discussed in section 2.5.6. Primer sets generally optimised at a concentration of 1.5 mM $MgCl_2$. This concentration was used unless otherwise stated.

2.5.5. Avoidance of contamination

In order to avoid any cross contamination from PCR products, reactions were set up in a designated area with 'PCR-clean' pipettes using plugged or positive displacement pipette tips. A gown, gloves, mob cap and face mask were worn. All products from PCR reactions were stored and analysed in a separate area from samples and PCR reagents. To further maximise this system all reagents for PCR were prepared and aliquoted outwith the Department of Veterinary Pathology.

The substitution of dUTP for dTTP within the PCR system facilitated the use of the enzyme uracil DNA glycosylase (Cambio) where contamination with PCR product was suspected. Prior to amplification, 1 unit of enzyme was added/100 μL reaction. The solution was heated to 37°C for 1 hour to allow breakdown of any contaminating product present. The initial denaturation in the standard PCR reaction, section 2.5.2., was increased from 5 to 10 minutes to inactivate the enzyme.

2.5.6. Analysis of PCR products

Products from PCR reactions were electrophoresed on 8% polyacrylamide gels (Table II.I.) on a mini gel apparatus (Table II.I.). Eight microlitres of sample with 2 μL of gel loading dye (Table II.II.) were loaded onto the gel and 400 ng of ϕX 174/Hae III marker (Gibco/BRL) were run on each gel as a size reference. The gel was run for 1 hour at 120 V then stained in a 0.5 μg/mL solution of ethidium bromide for 6 minutes. The DNA was visualised and the gel photographed as described previously (section 2.2.3.).

2.5.7. Electroblotting

Electroblotting was used for the transfer of PCR products from acrylamide gels to nylon membrane. Prior to blotting, gels were immersed in alkali buffer (Table II.II.) for 8 minutes to denature the DNA followed by immersion in neutralising buffer for a further 8 minutes. The gels were then placed in 1 x TAE transfer buffer (Table II.II.) before electroblotting. Transfer of DNA was performed using a mini-electroblotting apparatus (Bio Rad) and applying a constant 15 V across the gel for 1 hour. Following the blotting procedure DNA was bound to the membrane as described in section 2.2.4. Hybridisation with radiolabelled probe was performed to confirm the presence of specific product (section 2.4.4.).

2.6. CLONING OF PCR PRODUCTS

Prior to cloning, PCR products of interest were reamplified in a standard PCR reaction with the substitution of dTTPs for dUTPs. This reamplification was necessary as DNA containing dUTPs does not clone efficiently. Reamplification was performed by cutting a small piece of the PCR product of interest, amplified with dUTPs, from an ethidium bromide-stained polyacrylamide gel. The gel slice was placed in a PCR tube and mashed using a pipette tip. The PCR was then performed as standard (2.5.2.) but using dTTPs at a concentration of 200 μM and the

acrylamide gel slice containing the PCR product as template. The resulting PCR product was then used as described below.

Cloning of PCR products was performed using a commercially available kit (TA cloning kit, Invitrogen). The TA cloning kit takes advantage of the non-template dependent activity of the thermostable polymerase used in PCR which adds a single deoxyadenosine to the 3' ends of duplex molecules. These 3' A-overhangs are used to insert the PCR product into a vector which contains single 3' T-overhangs.

Following the manufacturers instructions a ligation reaction was performed utilising the following formula to determine the amount of PCR product to be ligated with 50 ng of supplied vector.

$$X \text{ ng PCR product} = \frac{[Y \text{ bp PCR product}][50 \text{ ng pCR}^{TM}\text{ Vector}]}{\text{size in bp of the pCR}^{TM}\text{ Vector}}$$

The ligation reaction was performed in an 11 μL volume containing 1 μL of 10x ligation buffer, 6 μL of sterile water, 2 μL of PCR vector, 1 μL of diluted PCR product and 1 μL of T4 DNA ligase. All components were supplied by the manufacturer. The reaction was placed at 12°C for 4 hours or overnight. The reaction was then transformed into One Shot™ competent cells following manufacturers instructions. LB agar plates (Table II.III.) containing kanamycin or ampicillin antibiotic at a concentration of 50 μg/mL were spread with 25 μL of X-Gal (40 mg/mL stock in dimethylformamide) to allow blue/white colony selection. Fifty and two hundred microlitre volumes of the transformed bacteria were spread onto the prepared plates and incubated overnight at 37°C. White colonies were selected for analyses and grown overnight in 5 mL of L-broth containing appropriate antibiotic. Plasmid preparations were performed on a small scale essentially as described in section 2.3.4. or by using commercially available purification columns (mini-Wizard preparations, Promega). Plasmids were screened for insertion of PCR product DNA by restriction endonuclease digestion and polyacrylamide gel electrophoresis (sections 2.2.2. and 2.2.6. respectively). Clones of interest were sequenced commercially (Alta Bioscience).

CHAPTER 3
LACK OF INVOLVEMENT OF THE BCL-2 GENE IN HODGKIN'S DISEASE

3.1. INTRODUCTION

bcl-2 is a proto-oncogene identified as a result of its involvement in the t(14;18)(q32;q21) chromosomal translocation which is characteristic of follicular lymphomas (Cleary & Sklar, 1985; Bakhshi *et al.*, 1985; Tsujimoto *et al.*, 1985). The translocation juxtaposes the *bcl-2* gene situated on chromosome 18 with the IgH gene on chromosome 14 and as a result the *bcl-2* gene is dysregulated and inappropriate levels of the *bcl-2* gene product are expressed (Graninger *et al.*, 1987; Seto *et al.*, 1988). Overexpression of Bcl-2 protein increases cell survival by protecting cells from undergoing programmed cell death or apoptosis (Hockenbery *et al.*, 1990). The translocation is associated with >75% of follicular lymphomas and 30% of diffuse lymphomas (Yunis *et al.*, 1987; Weiss *et al.*, 1987b). Approximately 60% of the translocations occur within a specific region designated the mbr region (Cleary & Sklar, 1985; Bakhshi *et al.*, 1987). The *bcl-2* gene is discussed in section 1.5. of this thesis.

Stetler-Stevenson *et al.* (1990), detected t(14;18)(q32;q21) involving the mbr of *bcl-2* in samples from 32% of HD patients using a PCR technique. If the translocations in these cases were present in the RS cells and inappropriate levels of the *bcl-2* gene product expressed, then this may contribute to increased survival of the tumour cells in HD. The EBV latent gene product LMP-1 has the ability to upregulate a number of cellular genes *in vitro*, including the proto-oncogene *bcl-2* (Gregory *et al.*, 1991; Henderson *et al.*, 1991). Many studies have reported that EBV is associated with at least one third of HD cases. In these cases EBV genomes are present in RS cells and EBV LMP-1 is expressed (Weiss *et al.*, 1987a; Anagnostopoulos *et al.* 1989; Herbst *et al,.* 1991; Pallesen *et al.*, 1991; Armstrong *et al.*, 1992b).

Two different mechanisms may therefore lead to the induction of Bcl-2 in RS cells. Upregulation of the *bcl-2* gene either by EBV infection or *bcl-2* translocation, may play a significant role in the pathogenesis of HD. To investigate this, the presence of t(14;18) involving the mbr was investigated in DNA samples derived from HD biopsy material of known EBV status. Bcl-2 expression was also investigated in a subset of cases.

3.2. MATERIALS AND METHODS

3.2.1. Samples

Using a PCR strategy, DNA samples from fresh or frozen biopsies from 50 patients with HD were investigated for the presence of translocations involving the mbr of *bcl-2* and J_H of the IgH gene. Samples were selected on the basis of availability of DNA and all had been assayed previously for the presence of EBV genomes. Twenty-seven cases were NSHD, 18 MCHD, 1 LDHD and 4 LPHD. Thirty one were EBV-associated (Armstrong *et al.*, 1992b). A subset of 23 cases was examined for the presence of IgH gene rearrangements, in 6 cases rearrangements were detectable by Southern blot analysis (Gledhill *et al.*, 1990).

3.2.2. PCR analysis

The primers used in the PCR were modified versions of those used by Stetler-Stevenson *et al.* (1990) (Fig 3.1.). Reactions were carried out in a 50 μL volume containing 1 unit of thermostable polymerase (Cambio, Cambridge, UK), 25 pmols of each primer, 1 x buffer supplied with the enzyme and 200 μM of each deoxynucleoside triphosphate (dATP, dGTP, dCTP, dTTP). Two micrograms of DNA were used as template and in order to confirm the presence of amplifiable DNA, identical aliquots were assayed using primers derived from the EBV genome or from the beta-globin gene. Forty cycles of amplification were performed in a programmable heat block using standard thermal cycling parameters (section 2.5.2.).

3.2.3. PCR sensitivity and analysis of product

DNA from a follicular lymphoma sample containing a *bcl-2* translocation detectable on Southern blot analysis, was used to determine the sensitivity of the PCR. Starting with 2 μg of DNA, six serial 10-fold dilutions were assayed. Fourteen replicates of the 10^{-5} dilution were also assessed. DNA from a positive HD sample (case 9) had a similar

titration performed, although in this instance, six replicates of each of the 10^{-4} and 10^{-5} dilutions were assessed.

Eight microlitres of products were electrophoresed on 8% polyacrylamide gels. Gels were subsequently stained and photographed, followed by a standard electroblotting procedure onto nylon membrane (sections 2.5.6. and 2.5.7.). Nylon filters were hybridised with 2.5 pmols of a ^{32}P-radiolabelled internal oligonucleotide probe specific for the *bcl-2* gene product (section 2.4.4.).

In each PCR experiment a negative control sample consisting of either water or placental DNA was assayed after every two HD samples. The majority of samples were assayed on more than one occasion. A positive HD sample, case 9, was also included in each assay.

3.2.4. Immunostaining

Frozen sections from 11 of the above cases were stained using an indirect immunoperoxidase technique and a monoclonal antibody to the Bcl-2 protein (bcl-2 100). RS cells in 7 of the 11 cases were EBV LMP-1 positive. Staining was carried out at the Department of Pathology, University of Edinburgh by Dr. A.S. Krajewski and colleagues. Frozen material from the remaining cases was not available for assessment of the expression of the *bcl-2* gene product.

3.2.5. Southern blot analysis

Southern blot analysis was used to determine whether the IgH or *bcl-2* loci were rearranged in samples from case 9. Probes used for hybridisation were a 3.5 kb probe to the IgH joining region (J_H) (Erikson *et al.*, 1982) and a 4.3 kb probe to the mbr of the *bcl-2* locus. Standard Southern blot and hybridisation protocols were performed in each of these experiments (sections 2.2.4. and 2.4.2.).

3.3. RESULTS

3.3.1. Polymerase chain reaction

Amplification products following PCR experiments were detectable on ethidium bromide-stained gels in the 10^{-4} dilution of the follicular lymphoma DNA sample. Following hybridisation, specific product was detected in 9 of the 14 replicates of the 10^{-5} dilution.

Three of the fifty samples contained t(14;18) translocations involving the mbr of the *bcl*-2 loci detected using the above primers. Samples from cases 9 and 66 gave rise to specific amplification products, clearly visible on gels stained with ethidium bromide (Fig 3.1.). On repeated analyses, case 2 gave rise to a weak signal after hybridisation with the specific oligonucleotide probe. Amplified products from the three cases were of a different size, thus ruling out contamination due to carry-over of PCR product.

3.3.2. t(14;18) positive cases

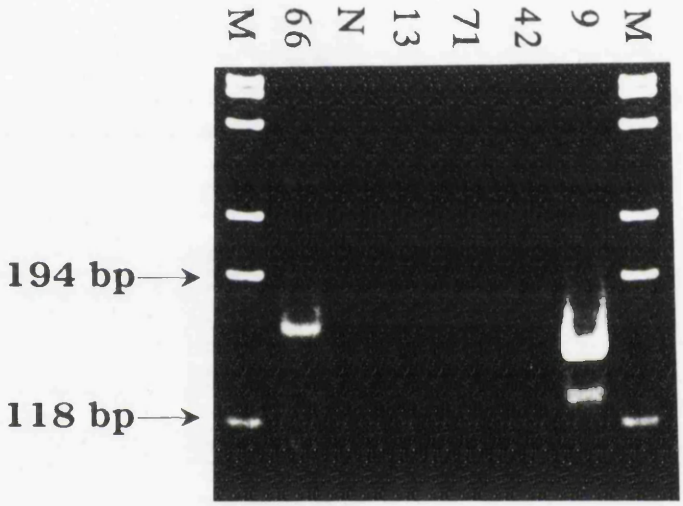
Case 2 was a 22 year old male with NSHD. The tumour was EBV-negative, LMP-1 negative and lacked TCR and IgH gene rearrangements on Southern blot analyses (Gledhill *et al.*, 1990).

Case 9 was a 50 year old female with a differential diagnosis of sclerosing mediastinal B-cell lymphoma and NSHD; on histological review 50% of the cells within this biopsy were considered malignant (Gledhill *et al.*, 1990). The tumour contained two IgH gene rearrangements detectable on Southern blot analysis (Gledhill *et al.*, 1991) and an IgH gene rearrangement detectable by PCR using IgH V and J region primers. Using Southern blot analysis rearrangement of the *bcl*-2 locus was not detected. The Southern blot analyses of case 9 are shown in Fig 3.2. The tumour was EBV-positive and LMP-1 expression was demonstrable (Gledhill *et al.*, 1991). The t(14;18) translocation was detectable in 3 of 6 replicates containing the 10^{-4} dilution of starting DNA, using the PCR methodology as described.

Fig 3.1. PCR products following polyacrylamide gel electrophoresis and ethidium bromide-staining. PCR was performed using IgH/*bcl-2* gene primers as detailed in the figure. Amplified IgH/*bcl-2* gene products are detectable in samples from cases 66 and 9. Samples from cases 13, 71 and 42 are negative. Detectable products are in the size range 118 bp to 194 bp.

M= ϕX 174/Hae III marker; N= negative control (water).

Fig 3.1. Amplification products of bcl-2/JH PCR



5' primer (bcl-2) :CTG TCG ACA CGG CCG TGT ATT ACT G
3' primers (J1-J5) :CTW ACC TGA RGA GAC GGT GAC C
(J6) :CAA AGG CCC TAG AGT GGC CAT T
W=A+T; R=A+G

Fig 3.2. Southern blot analyses of case 9 following restriction endonuclease digestion of sample DNA with Hind III enzyme. Fig **A** illustrates results obtained following hybridisation with an IgH (J_H) probe. Fig **B** illustrates results obtained following hybridisation with a *bcl-2* gene probe. Sample DNA is on the left hand side and placental control DNA on the right hand side of both figures **A** and **B**.

G= germline; R= rearrangement.

Fig 3.2. Southern blot analyses of case 9

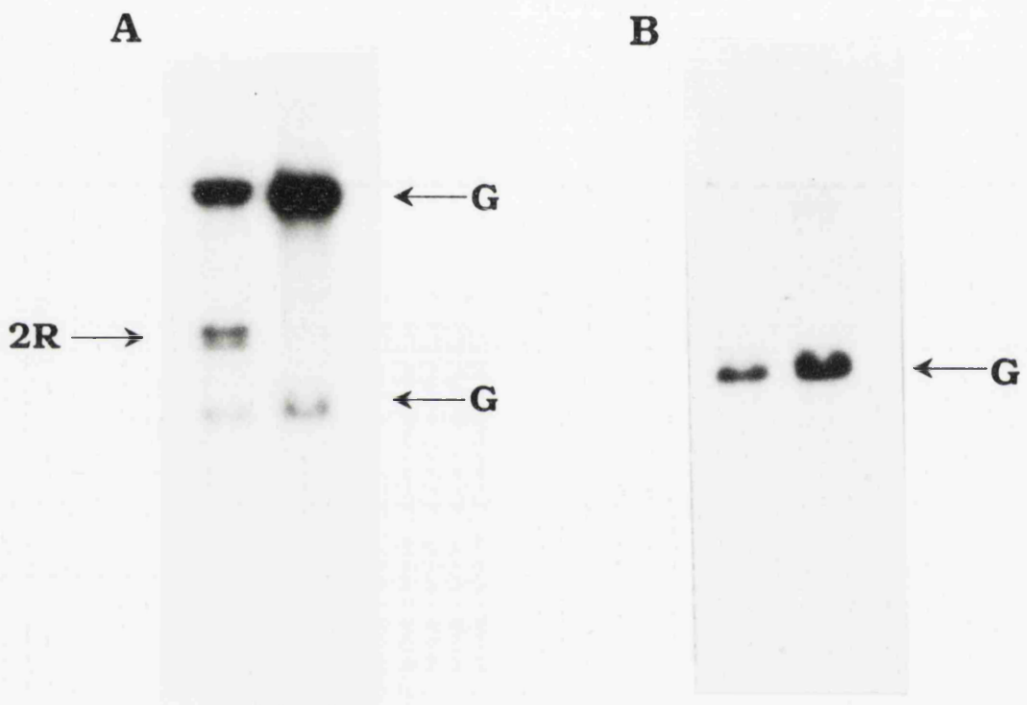


Fig A illustrates two IgH gene rearrangements present in case 9.

Fig B illustrates the germline configuration of the bcl-2 gene of case 9.

Case 66 was a 66 year old male with HDNS and the tumour was EBV positive on Southern blot analysis (Jarrett *et al.*, 1991).

3.3.3. Immunophenotyping

Reliable assessment of Bcl-2 expression was not possible in any of the three cases positive for t(14;18), as frozen material was not available. Reactivity with the Bcl-2 monoclonal antibody was however clearly detected within the RS cells of 5 of the 11 cases examined (Armstrong et al., 1992b). Strong cytoplasmic staining was present in these cases although this was less intense than that of the surrounding small lymphocytes. Three of seven EBV LMP-1 positive cases contained Bcl-2-positive RS cells. Control sections of normal lymph node showed staining of lymphocytes in paracortex and mantle zone but not in germinal centres.

3.4. DISCUSSION

In this study t(14;18) involving the mbr of *bcl-2* was detected in only 3 of 50 cases examined. In case 9 the diagnosis of HD was in doubt and therefore the significance of this result is unclear. In conclusion, this chromosomal translocation would appear to be an infrequent finding in HD. The *bcl-2* gene product was detected in the RS cells of a proportion of cases. The detection of t(14;18) did not correlate with EBV genome or LMP-1 positivity and Bcl-2 was present in the absence of the translocation.

Bcl-2 expression in RS cells, induced either by EBV or the t(14;18) chromosomal translocation, would appear not to be a critical event in the pathogenesis of HD.

The findings from our group have subsequently been confirmed by similar studies using PCR based methodology (Said *et al.*, 1991; Shibata *et al.*, 1990) or Southern blot analysis (Weiss *et al.*, 1987b; Athan *et al.*, 1992). One study reported the absence of the t(14;18) translocation involving both major and minor breakpoint regions of the *bcl-2* gene in a series of 26 HD cases (Louie *et al.*, 1991). In a further PCR study 4 of 21 HD cases examined contained translocations of this gene (Gupta *et al.*, 1992b). In this study the authenticity of the translocations was confirmed by nucleotide sequencing. More recently the translocation was detected in 7 of 74 cases of HD using paraffin-embedded material as PCR template (Reid *et al.*, 1993). In this study two of the positive cases were confirmed by sequencing and in one case the nuclei were sorted into large and small sub-populations to attempt to localise the translocation. In this case the t(14;18) signal was stronger in the large subpopulation of nuclei indicating that the translocation may be present in the RS cells. In a cytogenetic and PCR study rearrangement of the *bcl-2* gene was detected in 40% of 28 HD biopsy specimens examined, however only 1 patient had a t(14;18) translocation in RS cells (Poppema *et al.*, 1992).

The differences between the results are difficult to interpret. A possible explanation is that differences in patient referral or histopathological classification, may affect the HD cases available for examination. The

sensitivity of the assay in this type of study always comes under scrutiny. An evaluation of sensitivity in our assay indicates that if the translocation were present in greater than 1:100,000 cells it would have been detected. The tumour population in most cases will be greater than this, therefore, if the t(14;18) were present in these cells it would have been detected.

There is no direct evidence that the translocations detected by our group and others are present in the RS cell. It is necessary to clarify the cellular localisation of the translocation and in addition correlate these findings with Bcl-2 expression to ascertain the significance of the *bcl-2* gene in HD.

In case 9, if the translocation were present in the tumour population, which was estimated to be 50% of the cells, this should have been demonstrable on Southern blot analysis. After repeated attempts detection of this was not achieved, although the rearrangement involving the mbr of *bcl-2* in a follicular lymphoma sample was easily demonstrable. In addition, two IgH gene rearrangements were clearly detectable in case 9 following hybridisation to an IgH joining region probe using identical filters and similar methodology to that described above. Therefore, either the DNA fragment containing the translocation was present and migrating extremely close to the germline band making it difficult to distinguish the two, or, it was in a population of cells too small to detect by this method. A comparison of results from the PCR titration experiments performed on the follicular lymphoma sample and DNA from case 9, provides support for the latter interpretation. It is therefore possible that the clonal IgH gene rearrangements were present in a different population of cells from the *bcl-2* translocation. Limpens *et al.*, (1991) have reported the detection of t(14;18)(q32;q21) in benign lymphoid tissues with reactive hyperplasia, thus the translocation is not exclusively associated with malignant cells.

The t(14;18) translocations detected in the three samples in our study, may therefore have been present in the reactive component of the tumour. If this were the case, it is difficult to clarify the significance, if any, that this translocation has in the pathogenesis of HD.

As discussed previously Stetler-Stevenson *et al.*, (1990) reported the detection of the t(14;18) translocation in a proportion HD cases and implied that the gene may be involved in the pathogenesis of the disease. More recently Stetler-Stevenson's group have published a study where Bcl-2 expression in HD was investigated and correlated with the t(14;18) translocation and EBV positivity (Bhagat *et al.*, 1993). In keeping with the results that we have found, this study could not correlate the presence of Bcl-2 staining in the RS cells with the presence of the t(14;18) translocation, or EBV positivity. In contrast to their earlier findings the conclusion was drawn that the translocation may not play a significant role in HD. In a different study a comparison between LMP-1 positivity and Bcl-2 expression in RS cells of 53 cases of HD was made (Khan *et al.*, 1993). They concluded that EBV did not induce the expression of Bcl-2 in HD, however, the detection of Bcl-2 in the RS cells of 30% of cases led them to speculate that *bcl-2* may play a role in the EBV-negative cases. No investigation for the t(14;18) was made in this study.

The suggestion made by Reid *et al.*, (1993) that the rearrangement may be present in the RS cell population was not definitive and their results could not exclude the possibility that the translocation may be present in the reactive component of HD. Although Poppema *et al.*, (1992) found frequent abnormal karyotypes in RS cells the detection of t(14;18) was an infrequent finding. They concluded that the occurrence of a *bcl-2* rearrangement in HD most likely resulted from the presence of small bystander B-lymphocytes in the reactive component of the tumour tissue.

There is now a consensus viewpoint that the *bcl-2* gene has little significance in the pathogenesis of HD. Examination of the RS cells away from the reactive component of the tumour would assist in finally resolving this issue.

Localisation of the translocation or aberrant *bcl-2* transcripts to a particular cell type would be required. To do this, PCR *in situ* would assist in specifying which cell type is involved. Other possibilities include using single cell PCR techniques (Trumper *et al.*, 1993) or samples enriched for a particular cell type, to allow more specific studies

to be performed. One possible method of tumour cell enrichment is the use of SCID mice to allow selective outgrowth of a clonal population of cells outwith the confines of an immunological response.

When the above mentioned techniques are utilised successfully to study the RS cell then more specific and definitive conclusions can be made in studies such as this.

CHAPTER 4
TRANSPLANTATION OF HODGKIN'S DISEASE AND NON-HODGKIN'S LYMPHOMAS INTO SCID MICE

4.1. INTRODUCTION

The aetiology of HD and cellular origin of the RS cell remain unclear. Attempts to characterise the malignant cells in HD, the RS cell and the mononuclear Hodgkin's-like cells, have been unsuccessful. Despite extensive research utilising immunohistological and immunogenotypic analyses the lineage of the RS cell is unknown.

Difficulties are encountered while studying HD material due to the relatively small numbers of malignant cells present in the tumour mass. Attempts to purify RS cells have rarely been successful and cell lines derived from HD material have an uncertain relationship to the tumour of origin. New strategies for the study of HD material are therefore necessary to study the tumour population of HD in greater detail.

One possible approach is the transplantation of HD tumour material into immunodeficient animals. This technique has been used successfully in the study of other human tumours including NHLs. The use of SCID mice in the study of human malignancy is discussed in section 1.6. Recently SCID mice have been shown to support the growth of transplanted HD-derived cell lines (Kapp *et al.*, 1992). In two cases of HD, biopsy material was also successfully transplanted into SCID mice giving rise to EBV-positive human B-cell lymphoproliferations (Kapp *et al.*, 1992). At the present time EBV-negative lymphomas from HD material have not been described in the SCID mouse model.

In the present study the SCID mouse model was used for the study of both HD and NHLs. If the use of SCID mice could enable the outgrowth of a relatively pure population of cells originating from human tumour cells then the applications would be both exciting and novel. For instance in recent years the study of the t(14;18) translocation in HD has caused much controversy as it is not clear whether the translocation is present in the RS cell or in the reactive cellular infiltrate accompanying the tumour cell population (Chapter 3). Successful amplification of tumour cells in SCID mice would resolve this issue.

Human tumour cells expanded in the SCID mouse model could also be used to study viral involvement in HD and NHLs, using assays such as that described in more detail in Chapter 5.

The induction of EBV-driven lymphoproliferations in SCID mice has been described in section 1.6. EBV proliferates in the SCID mouse model after engraftment of EBV-positive human tumour material. Generally this is seen as a polyclonal expansion of B-lymphocytes although active replication of the virus has undoubtedly taken place.

EBV is thought to play an aetiological role in the pathogenesis of certain subgroups of HD but is infrequently associated with the NSHD group, particularly cases within the young adult age incidence peak. Epidemiological studies support the hypothesis that an infectious agent may be involved in the EBV-negative cases. A ubiquitous virus such as a herpesvirus would appear to be a candidate which would merit further investigation. If a different virus were present in HD, perhaps a new herpesvirus, then successful growth of tumour tissue in the SCID mouse model could give rise to valuable virus-rich material.

The aim of this study was to produce an animal model to investigate the histogenesis, immunology and molecular biology of HD, outwith the confines of an immunological response. Under these conditions selective outgrowth of the malignant cell population would allow more specific studies of HD to be completed. In order to clarify the relationship between tumours arising in the SCID mice and the original HD tumour it is necessary to establish the clonality of both tumour types.

Molecular techniques have been used to determine the clonality and lineage of NHLs. Clonal B- and T-cell populations can be distinguished in tumour samples by the analysis of Ig and TCR gene rearrangements by Southern blot analysis (Arnold *et al.*, 1983; O'Connor *et al.*, 1985; Flug *et al.*, 1987). In recent years the use of a PCR technique for the assessment of B-cell clonality has been described (McCarthy *et al.*, 1990; Dean & Norton, 1991). The procedure involves using standard PCR conditions with consensus primers to the V and J regions of the IgH gene. This technique has the advantage that it requires relatively small amounts of template DNA and the analysis is rapid thereby allowing

increased throughput of test samples. Clonality of the tumour tissue in this study was assessed using PCR analysis of IgH gene rearrangements and in a proportion of cases Southern blot analysis of the IgH gene. In the EBV-positive cases Southern blot analysis of the terminal repeat sequences of the EBV genomes present was used to assess clonality of the EBV-infected cells.

Analysis of the clonality of EBV-infected cells is possible due to the variable number of tandem repeat sequences present at each end of the linearised EBV genome (Pritchett *et al.*, 1975; Kintner & Sugden, 1979). In EBV-infected cells the linear termini are joined intracellularly to form covalently closed episomal DNA, which is present in multiple copies, in both cell lines and tumour cells. In clonal cell lines established at a multiplicity of infection (MOI) of less than 1, only one fused terminal restriction fragment is present (Kintner & Sugden, 1979). This gives rise to clonal EBV genomes which will contain identical numbers of terminal repeat sequences. In contrast, cell lines established at a higher MOI have multiple fused terminal fragments (Raab-Traub *et al.*, 1980). This results in a polyclonally EBV-infected cell population which will have variable numbers of terminal repeat sequences from each of the EBV genomes present.

The following sections discuss the attempt to engraft HD and NHLs into SCID mice and analysis of the resultant tumours.

4.2. MATERIALS AND METHODS

This project was undertaken in collaboration with Dr A.S. Krajewski (Department of Pathology, University of Edinburgh). The preparation of tumour cell suspensions and transplantation into SCID mice were performed at the University of Edinburgh. Additional analyses performed at the University of Edinburgh included immunophenotyping and *in situ* hybridisation. The methodology for these techniques is stated below. All the molecular analyses were carried out by myself at the LRF Virus Centre, University of Glasgow.

4.2.1. Preparation of tumour cell suspensions

HD and NHL lymphoma lymph nodes and one spleen were obtained from the Royal Infirmary and the Western General Hospital, Edinburgh. Forty four biopsy specimens were prepared for injection into SCID mice including 18 HD, 23 NHL and 3 reactive nodes. Fresh lymph nodes/spleen were cut into small fragments in RPMI/L-Glutamine (ICN) solution and filtered through a fine mesh strainer. The suspension was centrifuged and the pellet resuspended in 1 mL of the above medium.

4.2.2. Transplantation into SCID mice

SCID mice (strain CB-17) were obtained from a colony, bred and maintained in the Department of Medical Microbiology, University of Edinburgh animal house. SCID mice were injected between 6-10 weeks of age. Two hundred and fifty microlitres of tumour cell suspension, containing 0.7-7 x 10^7 cells, were given to each of four mice. Initially, different routes of injection were used (intraperitoneal, intravenous and subcutaneous) however after a few months, only the intraperitoneal route was employed.

Mice were sacrificed when there was detectable tumour or poor condition (10-20 weeks). To passage the tumour, a piece of the SCID tumour was made into a cell suspension and injected, as described above, into a further 4 mice.

4.2.3. Immunophenotyping and *in situ* hybridisation

Paraffin and frozen section immunophenotyping were carried out using a panel of T-cell, B-cell, monocyte and EBV markers. CD45 monoclonal antibodies were used to distinguish mouse and human tissue. EBER *in situ* hybridisation was used as an additional technique for the assessment of EBV status.

4.2.4. MOLECULAR ANALYSES

4.2.4.1. Extraction of HMW DNA

HMW DNA was extracted from the majority of the above cases using a standard Proteinase K and SDS digestion, followed by phenol/chloroform extractions to remove protein (section 2.2.1.). Some samples were extracted using the Stratagene DNA extraction kit, which eliminated the use of solvents in the extraction procedure. The kit was used following the manufacturers instructions and involved an initial digestion of tissue using Pronase enzyme, then removal of protein with an SDS and sodium chloride solution, followed by precipitation of the DNA in ethanol. In both extraction procedures, if DNA were degraded or not sufficient to allow spooling, then the sample was spun gently in a bench-top centrifuge (MSE) to pellet the fragments of DNA. Although these samples were unsuitable for Southern blot analysis, they were used in PCRs, in which intact genomic DNA was not required.

4.2.4.2. Southern blot analyses

Southern blot analysis (section 2.2.4.) was performed on the above samples, where intact DNA was available, for the investigation of EBV status and subsequent analysis of the clonality of EBV genomes. Ten micrograms of genomic DNA were restriction enzyme digested, electrophoresed and capillary blotted, using standard protocols (sections 2.2.2., 2.2.3. and 2.2.4., respectively).

To investigate the presence of EBV in tumour samples, DNA digested with the restriction enzyme Bam HI was hybridised with the EBV Bam HI-W probe under standard conditions (sections 2.4.1. and 2.4.2.). The Bam HI-W probe is a sensitive probe since it represents the major internal repeat sequence of the EBV genome. The clonality of EBV-positive tumours was assessed by hybridising DNA digested with Bam HI and Eco RI restriction enzymes with a 9 kb Eco RI-D insert probe (Arrand *et al.*, 1981) which includes the terminal repeat sequences of the EBV genome. Restriction endonuclease digestion with Eco RI/Bam HI enzymes and subsequent hybridisation with the Eco RI-D probe, as described in section 4.2.5.2 gives rise to a constant 6 kb band visible following autoradiography in all EBV-positive samples. An additional fragment or fragments corresponding to the various terminal repeat sequences are also detectable. Therefore, where clonal EBV is present a 6 kb band and another single band corresponding to the clonal terminal repeat sequence is present. If, however, a polyclonal EBV infection is present a ladder of bands representing the various EBV genomes is visualised in addition to the 6 kb band.

A proportion of cases were hybridised with an oligonucleotide probe (named Terminal 1) derived from sequences immediately 3' to the 5' terminal repeat sequence or with a sub-clone of EcoR 1-D termed pBSLY3 derived from unique sequences situated 5' to the 3' terminal repeats. Hybridisation and wash conditions were as described in section 2.4.4. Initially hybridised filters were exposed to autoradiographic film for 1 day, followed by a longer exposure as deemed appropriate.

4.2.4.3. PCR analysis of IgH gene rearrangement

In order to determine whether clonal B-cell populations were present within the tumour tissue a PCR was performed using DNA from original biopsy and SCID tumour specimens. The IgH PCR technique described in this section allows different clones to be distinguished on the basis of size of amplified fragments. A clonal B-cell population results in a single fragment of a specific size for that particular rearrangement. Therefore, if an identical clone were present in the original and SCID passaged tumour populations then an identical fragment size would be apparent.

The primers used in the reaction were common variable and joining region primers homologous to the V and J regions of the Ig H gene locus situated on chromosome 14. The primer sequences used are given in Table IV. I. PCR reaction conditions were optimised using a B-cell lymphoma DNA, shown to contain an IgH gene rearrangement on Southern blot analysis, as template (sections 2.5.3. and 2.5.4.).

Frozen material from the original tumour from case 1372 was not available therefore paraffin sections were used for the analysis of IgH gene rearrangements by PCR.

Table IV.I. IgH PCR primer sequences

Primer	**5'**								**3'**
1 (V)	CTG	TCG	ACA	CGG	CCG	TGT	ATT	ACT	G
2 (J1-J5)	CTC(T)	ACC	TGA	G(A)GA	GAC	GGT	GAC	C	
3 (J6)	CAA	AGG	CCC	TAG	AGT	GGC	CAT	T	

Bases in parenthesis show alternative bases at two positions

One microgram of sample DNA was used as template in a PCR reaction containing 1 μM primers (1, 2 and 3, shown above). All other reaction components were as stated in section 2.5.2. A negative control sample containing water was included after every two samples. A B-cell lymphoma sample was used as a positive control in each experiment. Standard PCR cycling conditions were performed (section 2.5.2.) and samples were analysed as discussed previously (section 2.5.6.).

In a proportion of samples Southern blot analysis for the detection of IgH gene rearrangement was performed as described in section 3.2.5.

4.2.4.4. Bcl-2 PCR and Southern blot analyses

Bcl-2 PCR identical to that described in section 3.2.2. was performed on case 1372 for the detection of the t(14;18) translocation and further assessment of the clonality of the passaged tumours in this case. In addition Southern blot analysis with a *bcl*-2 probe was performed on this sample.

4.2.4.5. D1S80 forensic DNA amplification kit for tumour typing

In this type of study involving numerous mice, it is necessary to clarify that passaged material has indeed arisen from the original injected tumour cell suspension. During the investigation of tumour samples in this study it became apparent that definitive testing was required on the SCID mice and original material to confirm the relationship between the two. It is possible to distinguish samples from different individuals by their unique allelic pattern utilising a PCR methodology.

The human genome contains large amounts of repetitive DNA sequences which are arranged as tandem repeat units. Tandem repeat units are termed Variable Number of Tandem Repeats, or VNTRs (Nakamura *et al.*, 1987). Discrimination between individuals is based mainly on the number of repeat units present and therefore VNTRs are considered "length" polymorphisms rather than sequence polymorphisms. Analysis of VNTR loci is useful for investigation of forensic samples (Budowle *et al.*, 1991) and in our case, for clarifying the relationship between an original tumour sample and subsequent passaged tumour material.

The VNTR marker D1S80 consists of repeat units, 16 bp in length (Kasai *et al.*, 1990) and has been localised by multipoint linkage analysis to chromosome 1p (Nakamura *et al.*, 1988.). The 29 known alleles determine 435 possible genotypes with a discrimination power of 95-98% on the population. A commercial kit is available which enables such typing to be performed (Cetus Corporation).

During the course of this study a tumour sample from an HDNS case appeared to be successfully passaged in the SCID mouse model and had an EBV-negative profile and an IgH gene rearrangement. This sample appeared potentially exciting as to date, EBV-negative tumours arising in SCID mice following transplantation of HD material have not been described. The results raised suspicion, however, when the IgH gene rearrangement derived from the passaged HD tumour was electrophoresed alongside an IgH PCR product from a centroblastic lymphoma-derived SCID tumour. Both IgH gene rearrangements appeared identical in size which seemed highly unlikely due to the nature of rearrangement of the IgH gene locus. DNA from the original

tumour and passaged tumour were typed for the VNTRs using the D1S80 forensic amplification reagent set following the manufacturers instructions.

The tumour derived in the SCID mouse did not match that of the original tumour sample but was identical to the VNTRs of the original tumour from the case of centroblastic lymphoma. This particular HD case was therefore removed from the study.

An incident such as the one arising in this situation is rare. However, the use of a simple PCR technique such as this can ensure that results obtained from SCID mouse tumours bear a direct relationship to original biopsy tissue. Subsequently, all DNA from original tumour samples and passaged tumour tissue were typed using this methodology to establish a firm relationship between human tumour and SCID mouse-derived human tumour material.

4.3. RESULTS

4.3.1. Development of lymphomas in SCID mice

Following injection and transplantation of tumour cell suspensions 5/18 HD and 6/23 NHL cases showed the presence of detectable tumour following a latent period of between 11-20 weeks. No tumours were detectable in mice following transplantation with reactive node tissue suspensions (3 cases). Usually only one animal per case developed lymphoma with the exception of case 2134 in which all animals injected developed a tumour. Table IV.II. states the diagnosis and case numbers of the original tumours which went on to develop lymphomas in the mice.

Table IV.II. Cases of HD and NHL transplanted into SCID mice

Diagnosis	Case No.
LPHD	1371
LPHD	1145
LPHD	2593
NSHD	2507
MCHD	1373
Diagnosis	**Case No.**
Centroblastic	1372
Centroblastic	2592
Centroblastic	2594
Immunoblastic	2620
Follicular CBCc	2506
Large cell anaplastic	2134(a)
Large cell anaplastic	2134(b)

CBCc=Centroblastic centrocytic

4.3.2. Necropsy findings

Only one animal developed a subcutaneous tumour. All other cases showed a large peritoneal mass invading liver, pancreas and bowel. Mesenteric nodal involvement was common with less frequent involvement of paraaortic and mediastinal nodes. Involvement of pulmonary thymic and cervical nodes was occasionally apparent. Tumours derived from case 2134 (anaplastic large cell lymphoma) showed splenic and gastrointestinal mucosal involvement which was not detectable in other tumours.

4.3.3. Results of analysis of transplanted HD tumours

The results of analysis of transplantation of individual HD tumour cell suspensions are listed in Tables IV.III. to IV.VII. A summary of the results obtained is given in section 4.3.4.

Case 1371

Table IV.III. Results of case **1371** (LPHD)

Biopsy	LMP	EBER	EBNA-2	EBV-BW	EBV-Clon	Ig-ICC	Ig-PCR
original	-ve	-ve	-ve	-ve	NP	-ve	-ve
1st pass	+ve	+ve	+ve	NP	NP	Aλ	R

LMP=LMP-1 staining; BW=Bam HI-W analysis; Clon=clonality analysis; ICC=Immunocytochemistry; NP=not performed; R=rearrangement; pass=passage.

The RS cells of case 1371 showed CD20 and CD15 positivity. The tumour was EBV-negative by LMP-1 and EBNA-2 immunostaining and EBER *in situ* hybridisation and in addition, EBV was not demonstrable by Southern blot analysis with the BamHI-W probe. No detectable IgH gene rearrangement was present in DNA from this original material. In contrast the passaged tumour was EBV-positive as determined by LMP-1, EBER and EBNA-2 results. A clonal IgH gene rearrangement was detectable by PCR, and both Ig expression and secretion were evident in the passaged tumour.

Case 1145

Table IV.IV. Results of case **1145** (LPHD)

Biopsy	LMP	EBER	EBNA-2	EBV-BW	EBV-Clon	Ig-ICC	Ig-PCR
original	-ve	-ve	-ve	-ve	NP	-ve	-ve
1st pass	+ve	+ve	+ve	NS	NP	Gκ (MA)	R

LMP=LMP-1 staining; BW=Bam HI-W analysis; Clon=clonality analysis; ICC=Immunocytochemistry; NP=not performed; NS=not satisfactory; R=rearrangement; pass=passage. Immunoglobulins in parentheses indicate the presence of minor populations.

RS cells from case 1145 were CD20 positive. EBV was not detectable by immunostaining or Southern blot analysis nor was there a rearrangement of the IgH gene demonstrable by PCR in the original tumour sample. The passaged tumour was shown to be EBV-positive by immunostaining and *in situ* hybridisation. An IgH gene rearrangement was demonstrable on PCR analysis and immunoglobulin expression and secretion were demonstrable. Passaged tumour material was diploid.

Case 2593

Table IV.V. Results of case **2593** (LPHD)

Biopsy	LMP	EBER	EBNA-2	EBV-BW	EBV-Clon	Ig-ICC	Ig-PCR
original	-ve	-ve	-ve	-ve	-ve	-ve	-ve
1st pass(a)	+ve	+ve	+ve	+ve	NP	MGλ	R
1st pass(b)	+ve	+ve	+ve	+ve	NP	MGκλ	R

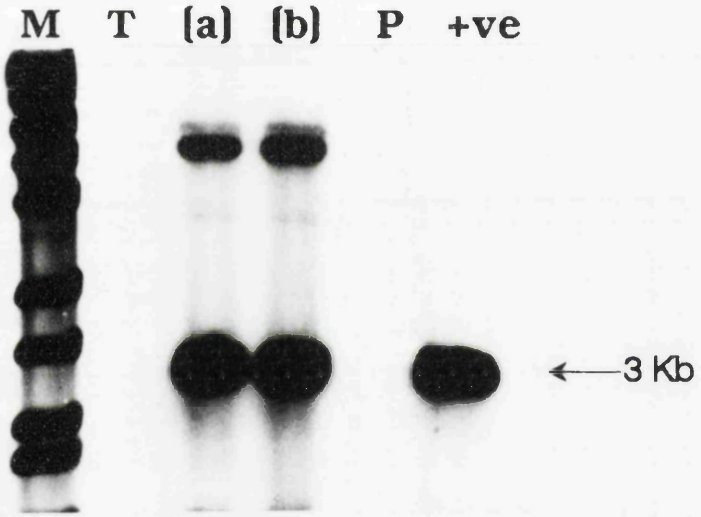
LMP=LMP-1 staining; BW=Bam HI-W analysis; Clon=clonality analysis; ICC=Immunocytochemistry; NP=Not performed; R=rearrangement; pass=passage.

RS cells in case 2593 were CD20 positive. Similar to cases 1371 and 1145, the original tumour was EBV negative and no IgH gene rearrangement was demonstrable by PCR. Both tumours arising in the SCID mice were EBV-positive by Southern blot analysis (Fig 4.1.), immunostaining and *in situ* hybridisation and contained IgH gene rearrangements as detected by the PCR technique. Immunoglobulin secretion and expression were demonstrable in both passaged tumours.

Fig 4.1. Southern blot analysis of case **2593** following restriction endonuclease digestion of sample DNA with Bam HI enzyme. DNA was hybridised with an EBV Bam HI-W specific probe. EBV-positive samples are identified by the presence of a 3 kb band as shown at the right hand side of the figure.

M= radioactive marker; T= original tumour sample; (a) & (b) denote SCID mouse tumours; P= placental control; +ve= EBV-infected B95-8 DNA.

Fig 4.1. EBV Southern blot analysis of case 2593



Original tumour from case 2593 is EBV-negative and both SCID tumours are EBV-positive as indicated by the 3 Kb band.

Both SCID mouse tumours were diploid. The IgH gene rearrangements detected in the mice were of different sizes, as shown in Fig 4.2.

Case 2507

Table IV.VI. Results of case **2507** (NSHD)

Biopsy	LMP	EBER	EBNA-2	EBV-BW	EBV-Clon	Ig-ICC	Ig-PCR
original	-ve	-ve	-ve	-ve	NP	-ve	-ve
1st pass	+ve	+ve	+ve	+ve	NP	Gλ (Mκ)	R

LMP=LMP-1 staining; BW=Bam HI-W analysis; Clon=clonality analysis; ICC=Immunocytochemistry; NP=Not performed; R=rearrangement; pass=passage. Immunoglobulins in parentheses indicate the presence of a minor population.

Both CD15 and CD30 were expressed by the RS cells in case 2507. EBV was not detectable in original tumour material by any of the above methods nor was a rearrangement of the IgH gene observed by PCR. The passaged tumour was positive for EBV by immunostaining, *in situ* hybridisation and Southern blot analysis. A clonal IgH gene rearrangement was present in the SCID tumour which also showed immunoglobulin expression and secretion. The passaged tumour was diploid.

Case 1373

Table IV.VII. Results of case **1373** (MCHD)

Biopsy	LMP	EBER	EBNA-2	EBV-BW	EBV-Clon	Ig-ICC	Ig-PCR
original	+ve	+ve	-ve	+ve	clonal	-ve	-ve
1st pass	+ve	+ve	+ve	+ve	oligo	Mκ (Gλ)	R

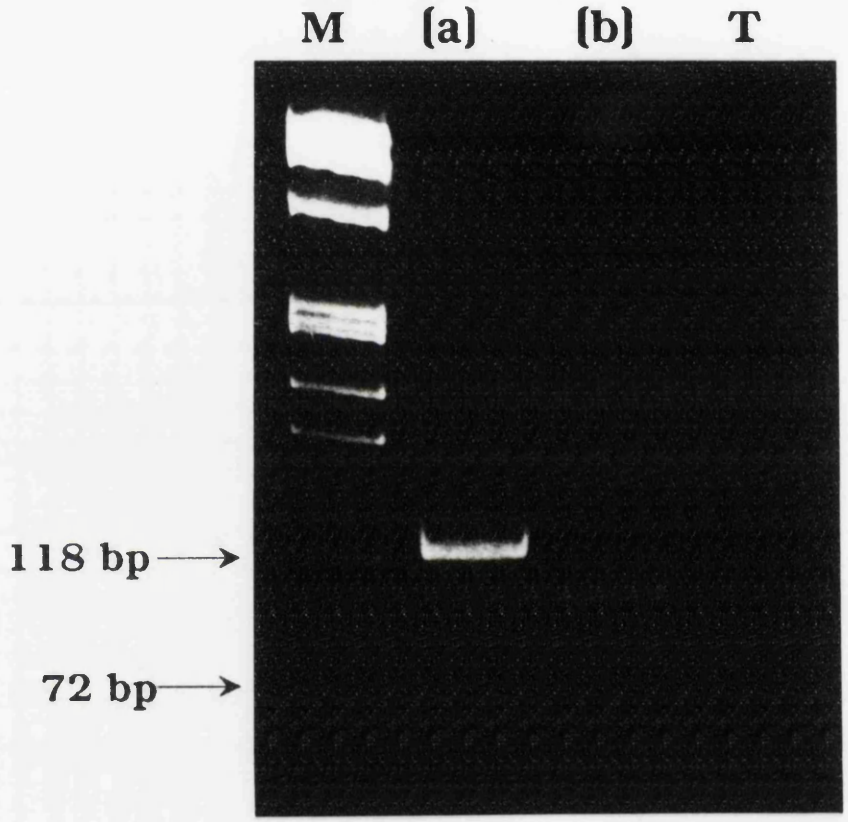
LMP=LMP-1 staining; BW=Bam HI-W analysis; Clon=clonality analysis; ICC=Immunocytochemistry; R=rearrangement; pass=passage; Immunoglobulins in parentheses indicate the presence of a minor population.

EBV was detectable in the original tumour material from case 1373 by LMP, EBER and Southern blot analysis. Examination of the terminal repeat sequences of this tumour revealed a clonal EBV-infected cell population. An IgH gene rearrangement was not detected in this sample

Fig 4.2. PCR products from case **2593** following amplification with IgH PCR primers as shown in Table IV.I. Products were electrophoresed on polyacrylamide gels and ethidium bromide-stained. A size reference is given in bp at the left hand side of the figure.

M= ϕX 174/Hae III marker; (a) & (b)= SCID mouse tumours; T= original tumour material.

Fig 4.2. IgH PCR analysis of case 2593



No IgH gene rearrangement was detectable in original tumour material. Both SCID tumours (a) & (b) have detectable rearrangements which differ in size.

by PCR analysis. The passaged tumour was also EBV-positive as shown in Table IV.VII. and hyridisation with a terminal repeat sequence probe was performed. The EBV in the SCID mouse tumour was oligoclonal in nature in contrast to the clonal EBV genomes in the original tumour. The dominant clonal EBV genome detectable in original tumour tissue differed in size to that of the passaged tumour. Passaged tumour was diploid.

4.3.4. Summary of the findings from transplanted HD tumour tissue

As described in Tables IV.III.-IV.VII. tumours developed in SCID mice following injection of tumour cells from 5 cases of HD. All of the tumours were high grade, large cell, B-cell lymphomas. As well as expressing B-cell antigens (CD19, CD20 and CD22) all cases expressed CD23 and CD43 with weak CD30 expression. All cases showed Ig expression usually with a marked predominance of a clone expressing a single heavy and light chain which was also present as secreted Ig in mouse serum. All of the SCID tumours had clonal IgH gene rearrangements, however none of the original HD tumours contained detectable rearrangements utilising the PCR technique. All rearrangements detectable by PCR were of differing sizes thus allowing distinction between the clones. In each of the tumours arising in the SCID mice expression of LMP-1, EBER and EBNA-2 was evident. Case 1373 (MCHD) was the only case in which LMP-1 and EBER expression were shown in the RS cells in the original biopsy material. In this case, clonal EBV was demonstrable by Southern blot analysis in the original sample however EBV in the passaged tumour was oligoclonal with no resemblance to the original clone. All of the tumours arising in SCID mice following transplantation of HD material were diploid.

4.3.5. Results of analysis of transplanted NHL tumours

The results obtained following the injection of cell suspensions from NHL cases into SCID mice are detailed in Tables IV.VIII.-IV.XIII. A summary of the results obtained is given in section 4.3.6.

Case 1372

Table IV.VIII. Results of case **1372** (CB lymphoma)

Biopsy	LMP	EBER	EBNA-2	EBV-BW	EBV-Clon	Ig-ICC	Ig-PCR
original #	-ve	-ve	-ve	NP	NP	Mκ	R*
1st pass (a)	-ve	-ve	-ve	-ve	-ve	Mκ	R*
2nd pass(b-d)	-ve	-ve	-ve	-ve	-ve	Mκ	R*
3rd pass (e-h)	-ve	-ve	-ve	-ve	-ve	Mκ	R*
4th pass (i-l)	-ve	-ve	-ve	-ve	-ve	Mκ	R*

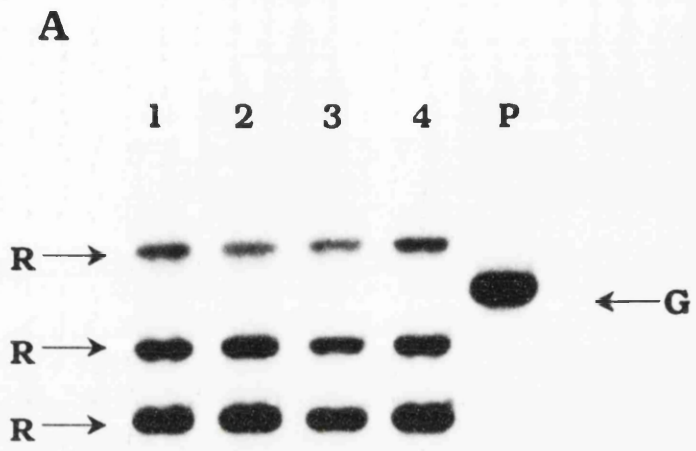
LMP=LMP-1 staining; BW=Bam HI-W analysis; Clon=clonality analysis; ICC=Immunocytochemistry; R*=identical size of rearrangement in original tissue and passaged tumour material; NP=not performed; # = No DNA available for Southern blot analysis; pass=passage.

Original material and passaged tumour tissue from case 1372 were consistently negative for the presence of EBV by the methods shown in Table IV.VIII. The tumour was successfully passaged 4 times with an identical IgH gene rearrangement present in the original tumour tissue and all subsequent passages. In addition identical Ig expression was detectable in both the original material and all passaged tissue, however no Ig secretion was evident. Although only one rearrangement was present in this case as detected by PCR, 3 rearrangements were detectable by Southern blot analysis in SCID tumours with the IgH gene J_H probe (Fig 4.3.(A)). The original tumour and SCID derived tumour also contained a *bcl*-2 translocation as detected by PCR; this translocation was detectable by Southern blot analysis in the SCID mice tumours on hybridisation with a *bcl*-2 fragment probe. Fig 4.3.(B) illustrates the presence of the *bcl*-2 translocation in passaged tumour material by Southern blot analysis.

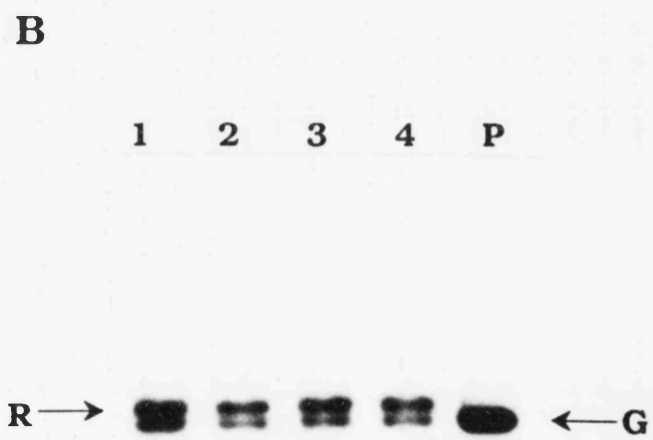
Fig 4.3. Southern blot analysis of case **1372** following restriction endonuclease digestion of sample DNA with Hind III enzyme. Fig **A** shows the result obtained following hybridisation of the DNA with an IgH joining region probe. Fig **B** illustrates the result obtained following hybridisation with a *bcl*-2 gene fragment probe.

R= gene rearrangement; G= germline; P= placental control; 1, 2, 3 and 4= representative samples of each of four passages of SCID mouse tumours.

Fig 4.3. Southern blot analyses of case 1372



Three IgH gene rearrangements were detectable in each of four passages of the SCID tumours from case 1372.



A bcl-2 gene rearrangement was detected in each of four passages of the SCID tumours from case 1372.

Case 2592

Table IV.IX. Results of case **2592** (CB lymphoma)

Biopsy	LMP	EBER	EBNA-2	EBV-BW	EBV-Clon	Ig-ICC	Ig-PCR
original	-ve	-ve	-ve	-ve	NP	Mκ	R*
1st pass	-ve	-ve	-ve	NP	NP	Mκ	R*

LMP=LMP-1 staining; BW=Bam HI-W analysis; Clon=clonality analysis; ICC=Immunocytochemistry; R*=identical gene rearrangement present in original tumour and passaged biopsy specimens; NP=not performed; pass=passage.

Both original material and SCID derived tumour material were negative for the presence of EBV by the above procedures. Both tumours had detectable IgH gene rearrangements of the same size as detected by PCR analysis, and exhibited identical Ig expression. Secretion of Ig was not apparent. Passaged tumour material was tetraploid.

Case 2594

Table IV.X. Results of case **2594** (CB lymphoma)

Biopsy	LMP	EBER	EBNA-2	EBV-BW	EBV-Clon	Ig-ICC	Ig-PCR
original	-ve	-ve	-ve	NS	NP	Gλ	R*
1st pass	-ve	-ve	-ve	-ve	NP	Gλ	R*

LMP=LMP-1 staining; BW=Bam H-I-W analysis; Clon=clonality analysis; ICC=Immunocytochemistry; R*=identical size of immunoglobulin gene rearrangement in original tissue and passaged tumour material; NP=not performed; NS=not satisfactory; pass=passage.

Original tissue and passaged tumour material were EBV-negative. Both tumours contained IgH gene rearrangements of the same size as detected by PCR analysis and showed similar Ig expression. No Ig secretion was detectable. Passaged tumour tissue was tetraploid.

Case 2620

Table IV.XI. Results of case **2620** (Immunoblastic lymphoma)

Biopsy	LMP	EBER	EBNA-2	EBV-BW	EBV-Clon	Ig-ICC	Ig-PCR
original	-ve	-ve	-ve	-ve	NP	Gκ	-ve
1st pass	-ve	-ve	-ve	-ve	NP	Gκ	-ve

LMP=LMP-1 staining; BW=Bam HI-W analysis; Clon=clonality analysis; ICC=Immunocytochemistry; NP=not performed; pass=passage.

In case 2620 both original material and passage tissue were EBV-negative. No IgH gene rearrangement was detectable in either tumour using PCR methodology. No DNA was available for assessment and comparison of IgH gene rearrangements by Southern blot analysis. Both tumours showed similar Ig expression and in the passaged tumour small amounts of secreted human IgG were detectable in mouse serum. The passaged tumour was aneuploid.

Case 2506

Table IV.XII. Results of case **2506** (CB/Cc- follicular lymphoma)

Biopsy	LMP	EBER	EBNA-2	EBV-BW	EBV-Clon	Ig-ICC	Ig-PCR
original	-ve	-ve	-ve	-ve	NP	Gκ	-ve
1st pass	+ve	+ve	+ve	+ve	NP	Gλ(MAκ)	+ve

LMP=LMP-1 staining; BW=Bam HI-W analysis; Clon=clonality analysis; ICC=Immunocytochemistry; NP=not performed; pass=passage. Immunoglobulins in parentheses indicate the presence of a minor population.

The original tumour material from case 2506 was EBV-negative however passaged tumour material was EBV-positive by immunostaining, *in situ* hybridisation and Southern blot analysis. No detectable IgH gene rearrangement was present in the original material, although a clonal IgH gene rearrangement was demonstrable in the passaged tumour by PCR. The Ig expressed in the original tumour differed from that of the passaged material which also showed Ig secretion. Passaged tumour material was diploid.

Case 2134

Table IV.XIII. Results of case **2134** (LC anaplastic lymphoma)

Biopsy	LMP	EBER	EBNA-2	EBV-BW	EBV-Clon	Ig-ICC	Ig-PCR
original (1)	<.01%	+ve	-ve	+ve	Clonal 1	A	-ve
1st pass (1a)	+ve	+ve	+ve	+ve	D*	Gλ (M)	R1
1st pass (1b)	+ve	+ve	+ve	+ve	D*	Mλ	R2
1st pass (1c)	+ve	+ve	+ve	+ve	D*	Gκ	R3
1st pass (1d)	+ve	+ve	+ve	+ve	D*	Mκλ (GA)	-ve
Biopsy	**LMP**	**EBER**	**EBNA-2**	**EBV-BW**	**EBV-Clon**	**Ig-ICC**	**Ig-PCR**
original (2)	-ve	+ve	-ve	+ve	Clonal 1	A	-ve
1st pass (2a)	+ve	+ve	-ve	+ve	D1*	A	-ve
1st pass (2b)	+ve	+ve	+ve	+ve	D1*	A	-ve
1st pass (2c)	-ve	+ve	-ve	+ve	D1*	A	-ve
1st pass (2d)	+ve	+ve	+ve	+ve	D*	A(M)	R4
1st pass (2e)	-ve	+ve	-ve	+ve	NS	A	-ve

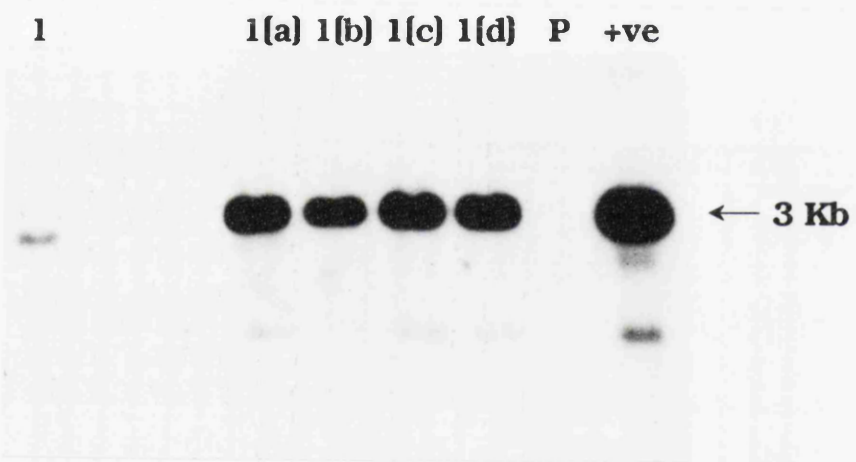
LMP=LMP-1 staining; BW=Bam HI-W analysis; Clon=clonality analysis; ICC=Immunocytochemistry; D*=Dominant clone with numerous linear or other episomal forms; D1*=identical size of dominant clone to original tumour specimens; R1-R4=IgH gene rearrangements detected by PCR of variable size. Immunoglobulins in parentheses indicate the presence of a minor population; pass=passage.

EBV was demonstrable in original tumour material from biopsy (1) by EBER *in situ* hybridisation and Southern blot analysis. Investigation of the EBV terminal repeat sequences in this sample revealed a clonal EBV pattern. An IgH gene rearrangement was not detected by PCR and sufficient DNA was not available for Southern blot analysis of the IgH gene, however, IgA was expressed in original tumour material. Injection of tumour material from biopsy (1) gave rise to tumours in 4 SCID mice. The presence of EBV was evident in all samples by Southern blot analysis (Fig 4.4.), EBER *in situ* hybridisation and LMP-1 and EBNA-2 immunostaining. On hybridisation with an EBV terminal repeat sequence probe, a dominant EBV genome was present in all passaged tumours with a ladder of other bands indicating the presence of additional episomes or linear forms. In 2 samples (1c and 1d) the dominant clone appeared similar in size to that of the original biopsy. Three SCID mice tumours contained clonal IgH gene rearrangements of different sizes when PCR analysis was performed. An IgH gene

Fig 4.4. Southern blot analysis of case **2134(1)** following restriction endonuclease digestion with Bam H1 enzyme. DNA was hybridised with a 3 kb EBV Bam H1-W probe.

1= original tumour sample; 1(a) - 1(d)= passaged SCID mouse tumours; P= placental control; +ve= EBV-infected B95-8 DNA.

Fig 4.4. EBV Southern blot analysis of case no 2134(1)



Original tumour 2134(1) and passaged SCID tumours are EBV- positive as indicated by the 3 Kb band.

rearrangement was detectable in the fourth SCID tumour by Southern blot analysis which differed in size to that of original tumour tissue. Both Ig expression and secretion were detected in these tumours however the Ig type was different in each of the cases and differed from that of the original tumour. Fig 4.5. illustrates the allelic pattern observed in original and SCID mouse tumour material following amplification with the D1S80 forensic kit as discussed in section 4.2.4.5.

EBV was also detected in original biopsy (2) from this case by EBER *in situ* and Southern blot analyses. Assessment of the EBV clonality showed the presence of an identical clonal EBV genome to that in biopsy (1). No IgH gene rearrangement was detectable in this sample by PCR although on Southern blot analysis IgH gene rearrangements were present. As in tumour (1), IgA expression was detected in original tumour material from biopsy (2). Five tumours developed in SCID mice following injection of original tumour cell suspension. All tumours were EBV-positive. Following hybridisation with an EBV terminal repeat sequence probe, 3 tumours (2a, 2b and 2c) had a demonstrable EBV clonal genome which was identical in size to that of the original tumour sample (Fig 4.6.). IgH gene rearrangements were not detectable in these samples by PCR, although on Southern blot analysis, IgH gene rearrangements identical to that of original tumour material were present in cases 2a, 2b and 2c. IgA expression was detectable in these SCID tumours which was identical to that expressed in original tumour material.

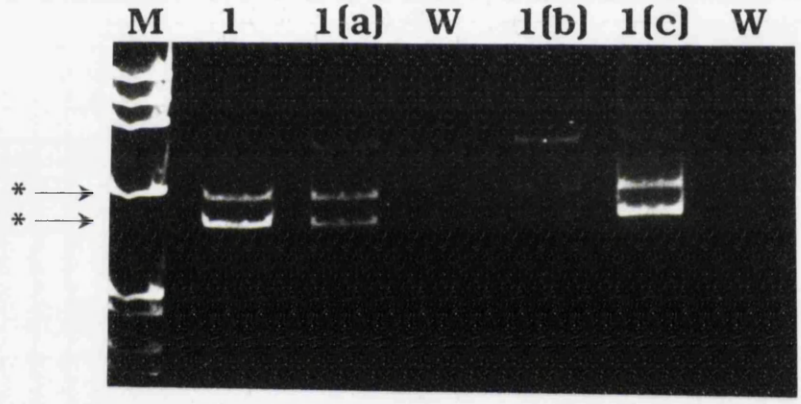
Biopsy 2d was EBV-positive however the dominant clone detectable on analysis of the terminal repeat sequences differed from that of original biopsy material. An IgH gene rearrangement was detectable in this sample by PCR and examination by Southern blot analysis showed that the rearrangements present also differed from original material. Both Ig expression and secretion were detectable in sample 2d and differed from that of original tumour specimen.

SCID tumour 2e showed EBER and not LMP-1 and EBNA-2 expression which was the same EBV gene expression pattern to that detected in the original biopsy material. No comparison of the terminal repeat sequence of the EBV genomes of this SCID tumour could be made as the DNA

Fig 4.5. Ethidium bromide-stained PCR products following amplification with the D1S80 forensic typing kit.

M= ϕX 174/Hae III marker; 1= original tumour 2134(1); 1(a)-1(c)= passaged SCID mouse tumours; W= negative water control.

Fig 4.5. D1S80 forensic typing of case no 2134(1)

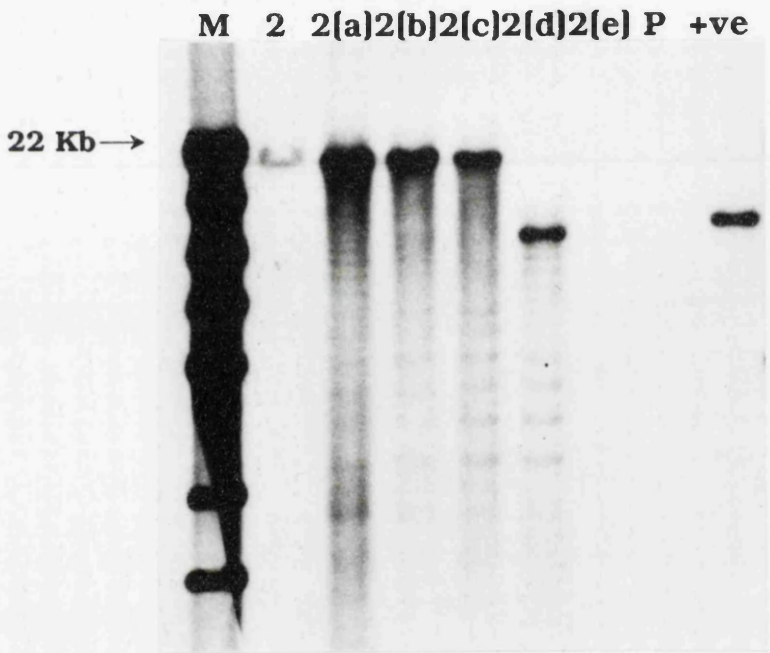


The symbol * illustrates the two bands common to original and passaged tumour samples indicating that the SCID tumours are derived from sample 2134(1).

Fig 4.6. Southern blot analysis of case **2134(2)** following restriction endonuclease digestion with Bam H1 enzyme. DNA was hybridised with an EBV terminal repeat sequence probe (Terminal 1).

M= radioactive marker; 2= original tumour 2134(2); 2(a)-2(e)= SCID mouse tumours; P= placental control; +ve= EBV-infected B958 DNA.

Fig 4.6. EBV terminal repeat sequence analysis of case 2134(2)



DNA from original tumour sample 2134(2) and SCID tumours 2(a), 2(b) and 2(c) contain a dominant EBV episome of identical size on Southern blot analysis. Sample 2(d) contains a dominant EBV episome which differs from original tumour material. Sample 2(e) is degraded.

sample became degraded during the course of the study. Similar to original tumour material, this sample had no detectable IgH gene rearrangements by PCR. A comparison of Southern blot analysis of the IgH gene was not possible however this tumour also showed similar Ig expression to the original sample.

4.3.6. Summary of the findings from transplanted NHL tumour tissue

Three cases of centroblastic lymphoma and one case of immunoblastic lymphoma produced tumours in SCID mice. These showed almost identical morphology and immunophenotype to the original tumours. In addition, the passaged tumours showed identical patterns of Ig expression and Ig gene rearrangement to the original biopsy specimens. These cases showed no detectable Ig secretion in mouse serum, except case 2620, an immunoblastic lymphoma, which showed small amounts of secreted human IgG. All of the tumours were tetraploid or aneuploid, and EBV was not detectable in any of the tumour samples.

One case of follicular lymphoma also produced a high grade lymphoma in a SCID mouse. Histologically this was a B-cell immunoblastic lymphoma which showed a different pattern of Ig expression and gene rearrangement to that of the original material. The secretion of human Ig in mouse serum was also detected in the SCID mouse from this case. This tumour was diploid, EBV was detectable by Southern blot analysis and EBV gene products were expressed.

Two biopsies from a case of large cell anaplastic lymphoma were transplanted into SCID mice. All mice injected with tumour cell suspensions developed lymphomas. All four tumours derived from the first biopsy and one of five tumours derived from the second biopsy were CD20-positive B-cell immunoblastic lymphomas expressing LMP-1, EBNA-2 and EBER. These tumours were diploid and all showed different patterns of Ig expression, and different IgH gene rearrangements to the original biopsy specimen. In contrast, four of the tumours derived from the second biopsy were CD20-negative large cell lymphomas containing multinucleated cells, similar to those in the

original biopsy. All were EBV-positive and in two of these tumours only EBER and not EBNA-2 and LMP-1 was expressed which was identical to the pattern seen in the original material. In one of these tumours a small number of LMP-1 positive cells were detected and the other tumour showed foci of LMP-1 and EBNA-2 positive cells. These tumours were tetraploid and contained an identical IgH gene rearrangement to that of the original material and identical IgA expression. One DNA sample was degraded and therefore Southern blot analysis of the IgH gene was not possible.

4.4. DISCUSSION

Following transplantation of tumour material from HD and NHL into SCID mice, three types of lymphoproliferative disorder were observed:

1. EBV-negative lymphomas derived from high grade NHLs. These tumours were aneuploid and showed phenotypic and genotypic findings which were identical to those of the original tumour specimens. In one case (1372) the tumour cells were successfully passaged four times.

2. EBV-positive lymphomas including those derived from a case of large cell anaplastic lymphoma. These tumours had phenotypic and genotypic findings which related to those found in original tumour material. These tumours showed EBER expression and minimal, or no, LMP-1 and EBNA-2. This pattern of EBV gene expression is that described in type I or type II latency as found in HD and in cases of large cell anaplastic lymphoma (Rowe *et al.*, 1992; Hamilton-Dutoit *et al.*, 1993).

3. EBV-induced lymphoproliferations derived from HD biopsies, one case of follicular lymphoma and some tumours from the large cell anaplastic lymphoma case. These tumours were diploid and showed clonal or oligoclonal IgH gene rearrangements with expression of EBER, LMP-1 and EBNA-2. This pattern of EBV expression corresponds to type III latency which is similar to that seen in LCLs and cases of lymphoma produced in SCID mice following injection of normal EBV-infected lymphocytes (Rowe *et al.*, 1992). Therefore, in contrast to the second category of EBV-positive tumours, these tumours are unlikely to have arisen from tumour cells within original tumour material. A plausible explanation is that the EBV-positive tumours within these SCID mice arose from normal B-cells within the tumour biopsy.

The results from this study clearly show that in some cases tumour material from NHL cases can be successfully propagated using the SCID mouse model. A proportion of these tumours were EBV-negative and therefore the virus could not have played a role in the development of the

SCID mouse tumours. Four EBV-positive tumours passaged from the case of large cell anaplastic lymphoma bore a direct relationship to original tumour material. SCID mice would therefore appear to be a useful tool for investigation of NHL tumour tissue.

In contrast to the above, transplantation of the RS cells contained within HD material into SCID mice appeared unsuccessful. In our experiments transplantation of HD tumour tissue into this model gave rise to EBV-driven lymphoproliferations. The only previous report of transplantation of HD into SCID mice was by Kapp *et al.*, (1993) in which 3/13 cases developed B-cell lymphoma following transplantation of tumour cell fragments. All 3 SCID mouse tumours were EBV-positive. In the latter study 3 categories were defined; (1) lymphoproliferative disease containing medium and large cell immunoblasts with some plasmacytoid cells, (2) lymphomas resembling anaplastic large cell lymphomas and (3) HD-like lesions containing RS-like cells surrounded by murine macrophages. The lymphoproliferative disease seen by this group corresponds closely to the polymorphous immunoblastic proliferations described in our study, however, no anaplastic large cell lymphomas or HD-like lesions were observed by us following injection of HD tumour suspensions into SCID mice.

It is well established that normal blood or tonsillar B-cells from individuals infected with EBV will, in most cases, produce B-cell lymphoproliferation following inoculation into SCID mice (Rowe *et al.*, 1991; Nakamine *et al.*, 1991, 1993). These cases appear to have a similar mature B-cell phenotype and EBV expression pattern to that seen by ourselves following transplantation of HD tumour material. It therefore appears unlikely that the HD-like tumours described by Kapp *et al.*, (1993) were derived from neoplastic cells in the original tumour material. A strong possibility is that these tumours were derived from EBV infection of normal B-cells within the reactive component of original tumour material. This is supported in our study by the normal diploid DNA content of these tumours.

A clear example of EBV lymphoproliferation arising from EBV-infected bystander cells was shown in HD case 1373 within this study. Original tumour material was shown to be EBV-positive, containing a clonal EBV

genome. In contrast, although the SCID mouse tumour was also EBV-positive, it contained oligoclonal EBV which differed in genome size from that of the original tumour. This highlights the important use of EBV as a clonal marker when analysing tissue samples. Unfortunately no detectable IgH gene rearrangements were present in original material from HD tissue samples and therefore could not be used for comparisons of clonality.

In conclusion, SCID mice do not seem to be a satisfactory model for the study of the neoplastic cells of HD at present. Tumours arising in SCID mice following injection of HD tumour cell suspensions appear to be EBV-driven lymphoproliferations. Further studies are required to resolve this issue using larger numbers of HD cases and clonotypic markers, such as those used in this study or cytogenetic markers, for comparison of original tumour tissue and passaged material. Perhaps in further studies the SCID mouse will prove to be an unsatisfactory model for the growth of HD tumour material due to the lack of a functional immune system. Cell-cell or cytokine interactions may be required for the growth of neoplastic cells in HD, however these functions are absent in SCID mice.

CHAPTER 5
A DEGENERATE PCR STRATEGY FOR THE DETECTION OF HERPESVIRUS SEQUENCES

5.1. INTRODUCTION

As previously described, epidemiological studies suggest an infectious aetiology in the young adult peak of HD in developed countries. Clonal EBV genomes have been detected, and localised to the RS cells, in approximately 40% of HD patients. EBV is associated with the paediatric and older adult cases of HD and is more frequently found in association with the HDMC as compared to the HDNS subtype. Young adult cases particularly of the HDNS subtype are non EBV-associated. It is within this group that strong evidence for an infectious aetiology exists. The relatively small numbers of malignant cells in the HD tumour mass have hampered research into both the characterisation of the RS cell and the search for novel infectious agents.

The primary aim of this study was to devise a PCR strategy to investigate whether known, or as yet uncharacterised herpesviruses, are present in the non EBV-associated HD cases, in particular young adult cases. A PCR suitable for this purpose must have the ability to detect herpesvirus sequences present at low copy number within the RS cells. To fulfil these requirements, the PCR must be sensitive enough to detect one viral genome present in 1% of the cells in a tumour mass. Since several herpesviruses may be present within a HD sample, either within the RS cell or the reactive infiltrate, a PCR capable of distinguishing different viruses is advantageous. Such a PCR would provide a useful tool not only in the search for herpesvirus sequences in HD, but also in other clinical conditions where viral involvement is suspected.

5.1.1. Degenerate primers

Each of the amino acids in a given protein is encoded by a 3 nucleotide combination termed a codon. Many amino acids have more than one codon and some such as leucine can be encoded by 6 different codons. Degenerate primers are a mixture of oligonucleotide sequences varying in base composition but with the same number of bases. In a PCR reaction, such primers may be substituted for primers of defined sequence to give specific gene amplification products. Degenerate

primers can be used when the amino acid sequence, but not the nucleotide sequence, is available for a particular protein.

Degenerate primers have been used successfully for the detection and cloning of a number of different genes e.g. the urate oxidase gene (Lee *et al.*, 1988) and the diabetes associated peptide (Girgis *et al.*, 1988). In both these examples, the sequence of the oligonucleotide mixtures was derived from an amino acid sequence where only a limited portion of the protein sequence was available.

Alternatively degenerate primers may be used when conserved nucleotide or amino acid sequences are available for a known gene family. Using these conserved sequences, new or as yet uncharacterised sequences related to a particular gene can be investigated. Mammalian and avian members of the hepadnavirus family were detected simultaneously using degenerate primers based on conserved amino acid sequence regions of the viral reverse transcriptase gene (Mack & Sninsky, 1988). In addition, degenerate primers have been used as a screening tool in the investigation of the association between human papillomavirus (HPV) and human colonic cancer. Utilising this strategy, McGregor *et al.*, (1993) confirmed the presence of HPV in a proportion of cases. A nested degenerate PCR, based on a relatively well conserved portion of the L1 capsid protein gene of genital HPVs, has also been used (Williamson & Rybicki, 1991). The two stage PCR was used to detect the presence of HPV in 13 out of 16 biopsies. Seven of the amplified products were sequenced, and one proved to be a previously uncharacterised HPV type.

Common to all herpesviruses is a glycoprotein referred to as gB in HSV-1 (section 1.3.2.). Utilising a degenerate PCR strategy, based on the sequences of two well-conserved pentapeptides of the gB or gB-like genes, Compton, (1990) isolated the gB gene of the feline herpesvirus 1 (FHV-1). The amino acid sequences of the known herpesvirus gB sequences were aligned and regions of conservation scored. A 23 amino acid region was identified that contained a unique sequence flanked by conserved pentapeptides. A set of primers was manufactured based on the preferred codon usage of serine and arginine (Aota *et al.*, 1988) but with both possible codons for tyrosine and glutamic acid. Restriction

enzyme sites were added to the 5' end of each primer to facilitate cloning and also improve primer duplex stability as previously described (Mack & Sninsky, 1988). A product of the predicted size of 85 bp (69 bp from the coding region plus 16 bp from the 5' extensions) was obtained. Following PCR amplification, the PCR gene product was cloned and sequenced. Employing a similar approach to that of Compton (1990), we designed a PCR strategy for the detection of herpesvirus gB sequences in DNA samples from clinical material.

5.2. OPTIMISATION AND VALIDATION

5.2.1. Materials

The sources of materials commonly used throughout this study are detailed in Table V.I. All other materials and their sources were as detailed in the text.

Table V.I. Sources of commonly used PCR materials

Materials	Sources
0.2 mL PCR tubes	Perkin-Elmer (Applied Biosystems)
PCR reagents:	All Perkin-Elmer (Applied Biosystems)
Amplitaq enzyme	
Nucleotides	
1.5 mM magnesium buffer	
Primers	Alta Bioscience
Metaphor agarose	Flowgen
TaqStart antibody	Clontech
Ampliwax beads	Perkin-Elmer (Applied Biosystems)
Sterile water	Gibco/BRL

All thermal cycling parameters were performed in a programmable heat block (Perkin-Elmer 9600, PCR machine) unless otherwise stated.

5.2.2. PRELIMINARY EXPERIMENTS

5.2.2.1. Degenerate gB primers

In preliminary experiments degenerate PCR primers, identical to those described by Compton (1990), were manufactured within the Department of Veterinary Pathology as described in section 2.5.1. The primers were titrated as described in section 2.5.3. using an HSV-1 template. On repeated occasions, with numerous different cycling parameters, no visible product was obtained following thermal cycling and polyacrylamide gel electrophoresis. EBV B95-8 DNA was used as template with the above primers and again no PCR product was detectable. Finally an EHV-4 template was chosen, as the primers appeared to cover the codon usage for this particular virus, and visible product was detected. It was thus clear that these primers do not have the ability to amplify all known herpesviruses, at least at the template concentrations tested. We require a PCR reaction which has the ability to detect any herpesvirus, and not selected sequences, within a clinical DNA sample, and therefore designed new primer sets to cover all known herpesviruses.

5.2.2.2. Primers containing inosine residues

Several experiments have suggested that inosine may be an "inert" base (Ohtsuka *et al.*, 1985; Martin *et al.*, 1985). Primers containing inosine bases have been used successfully to amplify genomic fragments for the generation of DNA probes in situations where only the peptide sequence was available (Aarts *et al.*, 1991). Inosine is usually inserted at base positions with 3- to 4-fold redundancy and therefore may be useful for the investigation of proteins with highly degenerate codons. The PCR strategy described in this chapter requires a highly degenerate primer set. An inosine residue incorporated into primers at a position containing a 4-fold degeneracy, would allow a reduction in the number of primers in the overall primer mixture.

A set of gB degenerate primers (section 5.2.2.1.) was manufactured with inosine bases substituted at positions containing 4-fold redundancies.

After repeated analysis no PCR product was detectable following gel electrophoresis and ethidium bromide-staining (section 2.5.6.). Inosine residues were therefore not used in future experiments.

5.2.2.3. Primers derived from herpesvirus polymerase genes

A set of degenerate primers based on the EBV polymerase gene was manufactured and tested using a strategy similar to that described by Teo *et al.* (1991). They used degenerate primers based on conserved amino acid sequences of 5 human herpesvirus polymerase genes to isolate a DNA probe for the DNA polymerase gene of HHV-6. We manufactured 3 primers, each with 16-fold degeneracy based on the EBV sequence. These primers were less degenerate than the primers used for the isolation of the HHV-6 polymerase gene. Combinations of the primers were tested using B95-8 DNA as template. Although the assay did work, certain primer sets only did so following the addition of 10% glycerol to the reaction. For our purposes a strategy was required which would allow uniform reaction and cycling conditions. We therefore decided that a strategy using gB degenerate primers was preferable.

5.2.3. RATIONALE FOR PCR WITH DEGENERATE gB PRIMERS

The sequences of the well conserved regions, corresponding to the pentapeptides CYSRP and EGQLG, were aligned for the herpesvirus gB and gB gene homologues (Table V.II.).

Table V.II. Alignment of two conserved pentapeptide regions of herpesvirus gB sequences.

VIRUS	N-terminal pentapeptide	C-terminal pentapeptide	Source
BHV-1	CYSRP	EGQLG	Lawrence *et al.*, 1986.
BHV-2	CYSRP	EGQLG	Hammerschmidt *et al.*, 1988.
EBV	CYSRP	EGQLG	Pellet *et al.*, 1985.
EHV-1	CYSRP	EGQLG	Whalley *et al.*, 1989.
EHV-4	CYSRP	EGQLG	Riggio *et al.*, 1988.
FHV-1	CYSRP	EGQLG	Spatz & Maes, 1993.
HCMV	CYSRP	YGQLG	Cranage *et al.*, 1986.
HHV-6 GS	CYNRP	LGQLG	Chou & Marousek, 1992.
HHV-6 Z29	CYNRP	PGQLG	Chou & Marousek, 1992.
HSV-1	CYSRP	EGQLG	Bzik *et al.*, 1984.
HSV-2	CYSRP	EGQLG	Bzik *et al.*, 1986.
ILTV	CYTRP	LGQLG	Griffin, 1991.
MCMV	CYSRP	SGQLG	Rapp *et al.*, 1992.
MDV	CYSRP	QGQLG	Ross *et al.*, 1989.
PRV	CYSRP	EGQLG	Robbins *et al.*, 1987.
SHV SA8	CYSRP	EGQLG	Borcher *et al.*, 1991.
VZV	CYSRP	EGQLG	Keller *et al.*, 1986.

BHV-1, Bovine herpesvirus-1; BHV-2, Bovine herpesvirus-2; EBV, Epstein-Barr virus; EHV-1, Equine herpesvirus-1; EHV-4, Equine herpesvirus-4; FHV-1, Feline herpesvirus-1; HCMV, Human cytomegalovirus; HHV-6, Human herpesvirus-6; HSV-1, Herpes simplex virus-1; HSV-2, Herpes simplex virus-2; ILTV, Infectious laryngotracheitis virus; MCMV, Murine cytomegalovirus; MDV, Marek's disease virus; PRV, Pseudorabies virus; SHV SA8, Simian herpesvirus SA8; VZV, Varicella-zoster virus.

The number of primers required to cover every possible codon usage of the aligned pentapeptides is 768 5' primers and 4096 3' primers. The amino acid codon usage of the pentapeptides is detailed in Tables V.III. and V.IV. From the outset it seemed likely that a single primer set, with this level of degeneracy, would not provide the necessary sensitivity. We therefore envisaged that multiple primer sets, each with more limited degeneracy, would be required.

An extensive set of experiments was performed to determine:

1. The maximum degeneracy that could be tolerated while maintaining the necessary sensitivity.
2. The optimal reaction and cycling conditions required.
3. A method of performing the assay which was economical in both time and expense.

These variables could not be considered independently and therefore, within many experiments, multiple comparisons were made. Since only one variable was changed in each comparison, many of the experiments appear large and complex.

The level of degeneracy that could be tolerated within the assay was assessed using an EBV viral template. An EBV template was also used to optimise cycling conditions and the sensitivity of the reaction before manufacturing the complete set of primers.

The rationales for choice of thermal cycling parameters can be conflicting and therefore various thermal cycling conditions were compared. Compton (1990) documented the importance of a low annealing temperature in the initial rounds of amplification, to allow the coding sequence of the small primers to anneal to target DNA. In subsequent cycles the annealing temperature was raised since incorporation of the restriction enzyme sites into the primer duplex increased the stability of the reaction. In contrast, a rationale for a high initial annealing temperature, followed by a gradual decrease in annealing temperature, also exists. A high annealing temperature in the initial cycles would allow specific priming to commence and a gradual decrease

Table V.III. Nucleotides required for all possible codon usage of amino acids C Y S/N/T R P

Conserved amino acid sequences

C			Y			S/N/T			R			P	
T	G	T	T	A	T	T	**C**	T	C	G	T	C	C
		C			C	A	**G**	C	A		C		
							A	A			A		
								G			G		
		2			2	2	4	4	2		4		

Numbers shown indicate the degeneracy at particular base positions. The bases highlighted in bold show only a 3-fold degeneracy at that particular position, however, due to technical limitations, a 4-fold degeneracy was synthesised. Only 2 bases from the codon for amino acid P were included. Table V.III. illustrates that a total of 768 5' primers are required to cover all possible codon usage of the amino acids shown.

Table V.IV. Nucleotides required for all possible codon usage of amino acids E/Y/L/P/S/QGQLG

Conserved amino acid sequences

E/Y/L/P/S/Q			G			Q			L			G		
	T	T	G	G	T	C	A	A	T	T	T	G	G	T
	C	C			C			G	C		C			C
	A	A			A						A			A
	G	G			G						G			G
	4	4			4			2	2		4			4

Numbers shown indicate the degeneracy required at the individual base positions. Table V.IV. illustrates that 4096 3' primers are required to cover all possible codon usage of the amino acids shown.

in temperature would enable more primer sequences to be used as specific product builds up. In view of these considerations thermal cycling programs were set up with, A: a constant annealing temperature, B: a low initial annealing, followed by a higher temperature and C: touchdown PCR, where annealing temperature is gradually decreased over a specified number of cycles.

A large number of degenerate primers was required for the assay. For ease of handling and to minimise outlay a PCR operable in a 96-well format was desirable.

Numerous experiments were performed in order to assess the optimal primer concentrations and buffering conditions of the PCR assay. These experiments are not detailed in this chapter however the optimal conditions are described in the experiments shown. The following sections describe the key experiments performed in the optimisation and streamlining of this assay. In addition, they describe the validation of the PCR for the detection of herpesvirus DNA sequences in DNA from clinical biopsy material.

5.2.4. EXPERIMENT 1
Toleration of degeneracy

As discussed in section 5.2.3., 768 5' and 4096 3' primers are required to cover all possible codon usage for the aligned pentapeptides. A set of degenerate primers would allow a reduction in the overall number of syntheses required. It appeared unlikely that a single set of highly degenerate primers would have sufficient sensitivity for our purposes. In order to assess the level of degeneracy that could realistically be tolerated in any individual reaction, an EBV-based primer set was manufactured. To save time and reduce cost a single EBV viral template was chosen for the following experiment.

5.2.4.1. EBV gB degenerate primers

A set of primers was manufactured, as described in section 2.5.1., based on the well-conserved pentapeptide regions CYSRP and EGQLG from the known gB sequence of B95-8 EBV DNA. Primers with an exact match to this sequence were manufactured, followed by primers containing varying amounts of degeneracy up to 128-fold, as shown in Table V.V. Degenerate bases were avoided at the 3' end of each primer. Five prime extensions containing restriction enzyme sites were added to each primer.

5.2.4.2. PCR conditions

Primer sets 106/112, 105/111, 104/110, 103/109, 102/108 and 102/107 (Table V.V.) were used at a concentration of 1 μM in individual experiments to assess the level of degeneracy that could be tolerated. Ten fold, serial dilutions from 2 μg - 0.2 pg of B95-8 DNA in water, were used as template. PCR was performed as described in section 2.5.2. and products obtained following thermal cycling were electrophoresed and visualised as described in section 2.5.6.

Table V.V. EBV gB degenerate primers.

5' EBV gB primers conserved amino acid sequences

Primer	Hind III	C	Y	S	R	P	Degeneracy
102	5'-TCA AAG CTT	TGY	TAY	TCN	CGN	CC-3'	64-fold
103		TGY	TAY	TCN	CGC	CC	16-fold
104		TGY	TAY	TCG	CGC	CC	4-fold
105		TGY	TAC	TCG	CGC	CC	2-fold
106		TGC	TAC	TCG	CGC	CC	none

3' EBV gB primers conserved amino acid sequences

Primer	EcoR I	G	L	Q	G	E	Degeneracy
107	5'-ATC GAA TTC	NCC	YAG	YTG	NCC	NT-3'	128-fold
108		NCC	YAG	YTG	NCC	CT	64-fold
109		NCC	YAG	YTG	TCC	CT	16-fold
110		NCC	YAG	CTG	TCC	CT	8-fold
111		NCC	CAG	CTG	TCC	CT	4-fold
112		GCC	CAG	CTG	TCC	CT	none

5' and 3' primers all 23-mers. N=A+C+G+T, Y=C+T.

5.2.4.3. Results

Specific EBV gB product was detectable, following ethidium bromide staining, using 2 ng of B95-8 DNA and the 64-fold degenerate primer combination, 102/108. No PCR product was detectable on ethidium bromide-stained gels when the most degenerate primer combination, 102/107 was used. In reactions containing the non-degenerate primer pair 106/112, PCR product was clearly demonstrable in the 0.2 pg dilution of B95-8 DNA.

The 64-fold degenerate primers 102 and 108 were chosen as candidates with which to perform further experiments to assess and optimise the sensitivity of the assay. Using 64-fold degenerate 5' and 3' primers the overall number of primers required was 12 and 64 respectively.

5.2.5. EXPERIMENT 2
Optimisation and sensitivity of 64-fold degenerate EBV gB primers

5.2.5.1. Template

To assess the sensitivity of the EBV gB degenerate primers 102/108 (Table V.V.) a sample containing a known number of viral genomes was required. The EBV-infected cell line Raji has a stable EBV copy number of 46 per cell and was therefore chosen as template for these experiments. Forty nanograms of Raji DNA were diluted, using serial 10-fold dilutions, to 0.4 ng of DNA in a background of 2 μg of human placental DNA. Placental DNA was used as diluent rather than water to mimic the effect of background DNA from cellular infiltrate which would be present when analysing HD DNA samples. The 0.4 ng dilution of Raji DNA in 2 μg of placental DNA is equivalent to 1 viral genome in 1% of the cells in a DNA sample derived from 3 x 10^5 cells. This was the sensitivity level required of the assay to allow detection of herpesviruses present within RS cells.

5.2.5.2. Hot Start PCR

Ampliwax beads were employed in the PCR reactions to facilitate a Hot Start. Hot Start PCR (Chou *et al.*, 1992) refers to the withholding of a critical reaction component until primers have attained a stringent annealing temperature. By performing a Hot Start PCR, primer-dimer formation and non-specific priming of oligonucleotides prior to thermal cycling are reduced and therefore more specific priming is attained. This is of critical importance when a low copy number of target sequence is present in background DNA. An Ampliwax bead was placed in the reaction tube containing reaction components excluding the primers. The reactions were heated to 70°C for 10 minutes to allow the wax bead to melt and cooled to room temperature to form a seal. Primers in buffer were added on top of the wax layer before commencing thermal cycling.

5.2.5.3. PCR conditions

PCR was carried out in a 50 μL volume containing 1 μM primers, 200 μM of each of the nucleotides (dGTP, dCTP, d ATP and dUTP), 1.5 mM magnesium buffer, and 1 U of Amplitaq DNA polymerase. Raji DNA dilutions as described in section 2.5.4.1. were used as template for the reactions. Hot Starts were performed (section 5.2.4.2.) on all of the above reactions with a 30 μL upper mix volume and a 20 μL lower mix volume to allow optimal mixing of components, after initial denaturation. In addition, a positive control containing 40 ng of Raji DNA diluted in 2 μg of human placental DNA as template and the less degenerate primers 103 and 109 was included in each of the experiments (section 5.2.3.1.). This control was included with and without the use of Hot Start to allow comparison of the two methods.

5.2.5.4. Thermal cycling conditions

Various thermal cycling conditions were tested in order to optimise the sensitivity of the assay. Prior to thermal cycling samples were denatured for 7 minutes at 94°C. Samples were ramped to 94°C over a 2 minute period and held at this temperature for 10 seconds. This was followed

by a 1 minute ramp to the annealing temperature and holding at temperature for 30 seconds. In experiments 2C, 2D, 2G and 2H, 5 cycles were performed at 1 temperature followed by 35 or 55 cycles at a different temperature. Annealing temperatures and cycle numbers were as detailed in Table V.VI.

Table V.VI. Thermal cycling conditions used in experiments 2A - 2H

Experiment No	Temp 1	Cycles	Temp 2	Cycles
2A	55°C	40		
2B	37°C	40		
2C	55°C	5	37°C	35
2D	37°C	5	55°C	35
2E	55°C	60		
2F	37°C	60		
2G	55°C	5	37°C	55
2H	37°C	5	55°C	55

Following thermal cycling, reactions were extended at 72°C for 7 minutes and finally brought to a temperature of 6°C. Amplification products were analysed as described in section 2.5.6.

5.2.5.5. Results

A PCR product of the expected size was clearly detectable on ethidium bromide-stained gels using 0.4 ng of Raji DNA and 60 cycles of amplification at 55°C (experiment 2E). Following 40 cycles of amplification with annealing at 55°C (experiment 2A), PCR product was only detectable down to the 4 ng dilution of Raji DNA. The increased cycle number in experiment 2E as compared to that of experiment 2A therefore increased the overall sensitivity of the reaction. In experiment 2F, where 60 cycles of amplification using an annealing temperature of 37°C were performed, PCR product was also detectable in the 0.4 ng dilution of Raji DNA. However numerous other non-specific bands were also clearly visible, suggesting that the non-stringent annealing had allowed the amplification of non-target sequences. As was observed in

experiment 2A, 40 cycles of amplification with an annealing temperature of 37°C resulted in a reduced signal intensity when compared with 60 cycles. Products from experiments 2B, 2C, 2D, 2G and 2H gave rise to weaker signals than those described above. Experiment 2E was repeated to confirm the result, blotted (section 2.5.7) and hybridised (section 2.4.4.) with an EBV-specific internal gB probe. The signal obtained following gel electrophoresis confirmed the original result and hybridisation to the probe confirmed the authenticity of the product. In all experiments the Hot Start PCR control with primers 103/109 gave an improved signal when compared to the non Hot Start PCR.

In conclusion the assay conditions used in experiment 2E, with 60 cycles of annealing at 55°C, appears sensitive enough to detect 1 copy of a herpesvirus if present in 1% of the cell population in 2 μg of template DNA (Fig 5.1.). In addition, Hot Start PCR improved overall sensitivity with the primer set 103/109.

5.2.6. EXPERIMENT 3
Optimisation of PCR product detection

Hybridisation is not possible when searching for new or as yet uncharacterised viruses when there are no conserved sequences internal to the primer sequences. In this experiment various methods for the detection of PCR products were compared in an attempt to maximise the sensitivity of the assay.

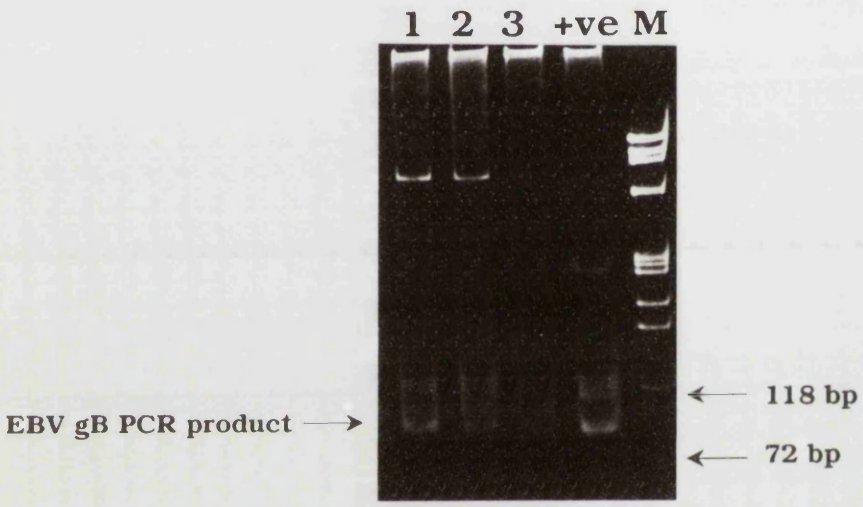
5.2.6.1. Captagene GCN4 kit

The Captagene GCN4 kit (British Bio-technology Products Ltd.) is an enzyme-linked assay for the detection of amplified target DNA. One primer within the reaction is biotinylated and the second incorporates a specific 12 bp recognition sequence for the dsDNA binding protein GCN4 (Hinnebusch, 1984). Amplified dsDNA containing this specific recognition sequence is captured in the wells of a microtitre plate via solid-phase GCN4 fusion protein. The presence of amplified DNA can then be detected by a biotin-streptavidin peroxidase colorimetric

Fig 5.1. Sensitivity of EBV gB degenerate primers. Template used was Raji DNA diluted in 2 μg of human placental DNA as shown below.

M= ϕX 174/Hae III marker; 1= 40 ng; 2= 4 ng; 3= 0.4 ng. Samples 1-3 all amplified with primers 102/108. +ve= 40 ng amplified with primers 103/109.

Fig 5.1. Sensitivity of EBV gB degenerate primers



EBV gB PCR product is detectable in each of the samples using primers 102/108.

reaction. The technique has been used successfully for the detection of HIV sequences in acquired immunodeficiency syndrome (AIIDS) patients (Kemp *et al.*, 1990). Such a system would be an ideal screening method for the detection of specific PCR product within this assay without the need for gel electrophoresis. Samples which appeared positive using the GCN4 methodology could then be further analysed.

A set of EBV gB degenerate PCR primers containing the biotinylated group and GCN4 recognition sequence were synthesised. Following amplification the GCN4 kit was used for the detection of PCR product. A set of negative controls, containing placental DNA as template, were amplified under the same conditions as the test sample, to assess non-specific background. Background within the negative controls was at an unacceptably high level. This particular screening method was therefore considered inappropriate for the detection of gB PCR products.

5.2.6.2. Fluorescein-labelled primers

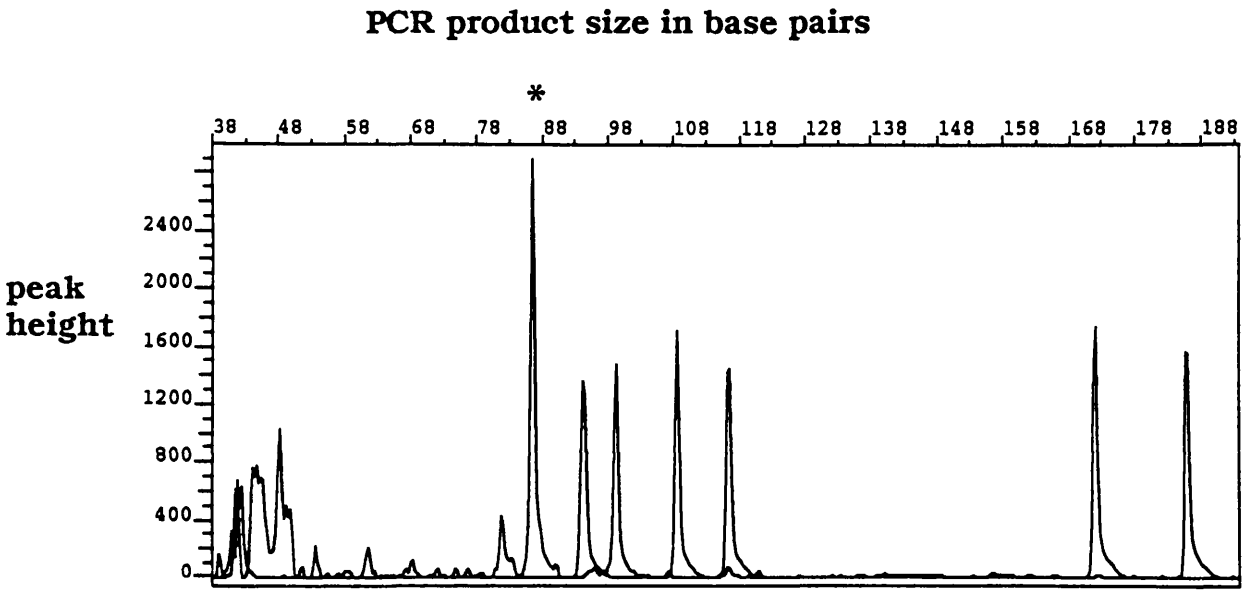
Utilising an automated sequencer, products of PCRs may be analysed, without ethidium bromide staining, by scanning for a specific dye-label such as fluorescein. Manufacturers have claimed greater than a 100-fold increase in detection sensitivity using this method compared to ethidium bromide-staining. Primers identical to 102/108 (section 5.2.3.1.) were manufactured with fluorescein groups on the 5' end of the primers. Test samples were amplified using fluoresceinated primers and analysed on an automated sequencer (Applied Biosystems). Results suggested that this would be the preferred method of analysis if the apparatus were available. The system enables accurate sizing and good visualisation of PCR product as shown in Fig 5.2.

A comparison was made between fluoresceinated and non-fluoresceinated primers to determine the effect of the fluorescein group on the sensitivity of the reaction. Ethidium bromide-stained gels were utilised in this analysis. In direct comparative experiments fluoresceinated primers performed inconsistently compared to the non-fluoresceinated primers. It was therefore decided to continue to use non-fluoresceinated primers in optimisation experiments.

Fig 5.2. Analysis of fluoresceinated PCR products using the Applied Biosystems 373A automated sequencer. EBV-specific gB PCR product is 87 bp as shown in the figure.

Size markers: 43 bp, 94 bp, 109 bp, 116 bp, 172 bp and 186 bp.

Fig 5.2. Automated analysis of fluoresceinated PCR products



* Indicates the expected size of EBV PCR product of 87 bp.

5.2.6.3. A nested PCR strategy for the analysis of small samples

A nested PCR strategy was designed to increase template DNA from small samples for subsequent use in the gB PCR. One 5' (A) and one 3' (B) primer containing a string of Ns followed by 5 specific bases for the 5 initial coding bases of the EBV primers 102 and 108 were manufactured (Table V.V.). The primer sequences are shown below.

5' Primers			
A	5'- NNNN NNN	**TGY TA**	-3'
102	5'-	**TGY TA**	Y TCN CGN CC-3'
3' Primers			
B	5'- NNNN NNN	**NCC YA**	-3'
108	5'-	**NCC YA**	GYTG NCC CT-3'

Bases boxed in bold indicate the 5 bases specific for the 5' coding sequence of primers 102 and 108 respectively. N=A+G+C+T, Y=C+T

We hypothesised that first round amplification with primers A and B may increase the specific gB template in the DNA sample due to the presence of the 5 bases homologous to the gB region of interest. To test this hypothesis 0.4 ng of Raji DNA, diluted in water, were amplified with 1 μM primers A and B in a standard PCR reaction (section 2.5.2.). Samples were denatured at 94°C for 5 minutes. Fifty cycles of amplification were then performed by denaturing samples at 94°C followed by an annealing step at 37°C for 1 minute. The products of the reaction were analysed as described in section 2.5.6.

No specific product was detectable on an ethidium bromide-stained gel although, as expected, a smear was visible indicating the presence of products of variable size. The PCR product was diluted and a 1/200th of the total PCR was used as template in a further PCR reaction, with 2 μM primers 102/108 and all other components as previously stated (section 2.5.2.). A control sample containing 10 ng of EBV-infected Raji DNA was included. Samples were denatured at 94°C for 5 minutes, followed by 60 cycles of amplification by denaturing at 94°C for 30 seconds and

annealing at 65°C for 1 minute. Products of the reaction were analysed as described in section 2.5.6.

Product of the expected size of 87 bp was clearly detectable on an ethidium bromide-stained gel from the control sample containing 10 ng of Raji DNA. In the test samples product of the correct size was also clearly detected (Fig.5.3.). Therefore sufficient template was generated in the first PCR reaction, utilising primers A and B and 0.4 ng of Raji DNA, to use in 200 individual PCR reactions for subsequent investigation with degenerate gB primers.

This particular strategy will be advantageous in situations were only limited amounts of template DNA are available for the assay. Due to the large primer set used in the gB assay substantial amounts of DNA are required as template. In a situation where purified RS cell populations are used for the assay only a small amount of template DNA will be available. The use of the nested PCR strategy may increase specificity of the reaction and allow a decrease in the amount of template required.

5.2.7. EXPERIMENT 4
Assessment of primer purification methods

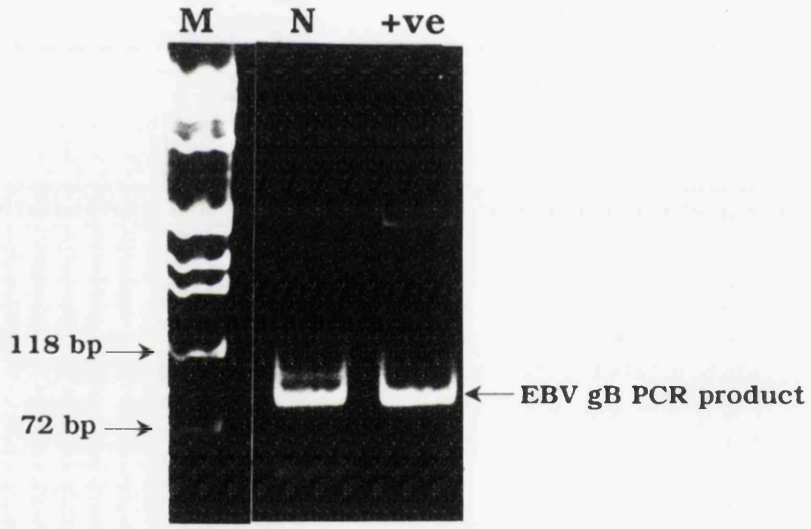
Before a large primer set was manufactured a comparison of various primer purification methods was made using the EBV gB degenerate primers. Cartridge-purified, polyacrylamide gel electrophoresis (PAGE)-purified and non-purified primers were manufactured with identical sequence to the primers 102 and 108 (section 5.2.3.1.). Primers were used at a concentration of 2 μM. One microgram of B95-8 DNA was used as template and all other components and cycling conditions were as described in section 2.5.2. Amplified products were analysed as described in section 2.5.6.

Cartridge-purified primers gave a distinctly stronger signal when compared to non-purified and PAGE-purified primer combinations. The results obtained in this experiment suggest that the use of cartridge-purified primers at a concentration of 2 μM is optimal.

Fig 5.3. Ethidium bromide-stained EBV gB PCR products following nested PCR analysis as described in section 5.2.6.3.

M= ϕX 174/Hae III marker; N= nested PCR product; +ve= 10 ng Raji DNA.

Fig 5.3. Nested PCR analysis of EBV



EBV PCR product is clearly detectable following nested PCR analysis.

5.2.8. EXPERIMENT 5
Comparison of 64-fold and 128-fold degenerate EBV gB primers

As we intend to search for as yet uncharacterised viruses our strategy has the need to cover every possible codon usage for the conserved amino acid sequences. If 64-fold degenerate 5' primers were used then 12 5' primers would be required. To minimise expense and increase practicality, a comparison was made between the existing 64-fold and a new 128-fold degenerate 5' primer. A 128-fold degenerate 5' primer would reduce the number of 5' primers required to only 6. Mismatch between target and primer are not well tolerated at the 3' end of the primer and therefore degeneracies were placed towards the 5' end of the unique primer sequence. In addition, by keeping degeneracy away from the 3' end of the primer different viruses may be distinguished by their preferential primer usage.

5.2.8.1. Primer sets

A set of primers were manufactured to allow comparisons of the 128-fold degenerate and 64-fold degenerate 5' primers to be assessed. The sequences of the 5' primers used were as detailed below. The 3' primer sequence was oligonucleotide 108 (Table V.V.).

64-fold degenerate 5' primer - 5' TCA AAG CTT TGY TAY TCN CGN CC 3'
128-fold degenerate 5' primer - 5' TCA AAGCTT TGY TAY WNN CGC CC 3'
N=A+G+C+T, W=A+T and Y=C+T.

5.2.8.2 Cycling conditions

Reactions were performed in a 50 μL volume, containing 2 μM of each primer with all other components as described in section 5.2.4.3. A Hot Start was performed throughout (section 5.2.5.2.). Template used was Raji DNA dilutions in placental DNA as described in section 5.2.5.1. Various thermal cycling profiles were performed using each primer pair to assess the optimal primer set and preferred cycling conditions. Reactions were denatured for 5 minutes at 94°C prior to thermal cycling.

In each cycle the reactions were denatured at 94°C for 30 seconds and then annealed as specified below. In experiments 5C and 5D a "touchdown" PCR was performed in which the annealing temperature was dropped by 0.5°C per cycle for a specified number of cycles. In experiment 5C a touchdown strategy was used over the first 20 cycles followed by a constant temperature of 55°C for 40 cycles. The touchdown was used to test whether stringent annealing in initial cycles would allow specific priming of product, followed by a lower annealing temperature which would increase the sensitivity of the reaction. The temperatures and cycle numbers were as detailed in Table V.VII.

Table V.VII. Thermal cycling conditions used in experiments 5A-5D

Experiment	Temp 1	At temp	Cycles	Temp 2	At temp	Cycles
5A	55°C	1 min	60			
5B	65°C	1 min	60			
5C	**65°C-55°C**	1 min	20	55°C	1 min	40
5D	**65°C-**	1 min	60			

Temperatures boxed in bold indicate touchdown thermal cycling.

5.2.8.3. Results and conclusions

Following acrylamide gel electrophoresis and ethidium bromide-staining of amplified PCR product (section 2.5.6.), the intensity of the EBV gB product using the primer sets and cycling conditions was compared. The touchdown PCR as described in experiment 5D did not give rise to detectable PCR product with either of the primer combinations. After analysis using conditions described in experiment 5C, PCR product was demonstrable in the 0.4 ng dilution of Raji DNA in the reaction containing the 128-fold degenerate 5' primer but not in the reaction containing the 64-fold degenerate primer combination. Both primer sets resulted in detection of product in 0.4 ng of Raji DNA, using the cycling conditions described in 5A and 5B. The 65°C annealing temperature gave rise to less non-specific amplification products than 55°C annealing and the intensity of specific bands was comparable.

In conclusion, the 128-fold degenerate 5' primer gave rise to PCR product signal intensity which was comparable with that obtained using the 64-fold degenerate primer. By changing from a 64-fold, to a 128-fold degenerate 5' primer, only 6 5' primers were required for the assay. The reduction in primer number brought the assay a step closer to a 96-well microtitre format. Although an annealing temperature of 65°C gave a more specific result this was considered too stringent an annealing temperature to ensure detection of all possible viral sequences. A comparison of annealing temperatures was performed in further experiments utilising different viral templates.

5.2.9. EXPERIMENT 6
Testing of known viral templates with gB degenerate primers

In order to test whether the conditions optimised for the EBV template in previous sections would work equally well on other viral templates, the primers detailed in Table V.VIII. were manufactured.

Table V.VIII. Sequences of primers 6A-6H.

128-fold degenerate 5' primers all 23-mers

Virus	5' Primer	Hind III
HSV-1	6A	5' TCA AAG CTT TGY TAY WNN CGC CC 3'
PRV	6B	TCA AAG CTT TGY TAY WNN CGC CC
VZV	6C	TCA AAG CTT TGY TAY WNN CGT CC
EHV-4	6D	TCA AAG CTT TGY TAY WNN CGC CC

64-fold degenerate 3' primers all 23-mers

Virus	3' Primer	Eco R1
HSV-1	6E	5' ATC GAA TTC NCC NAR YTG CCC CT 3'
PRV	6F	ATC GAA TTC NCC NAR YTG GCC CT
VZV	6G	ATC GAA TTC NCC NAR YTG GCC CT
EHV-4	6H	ATC GAA TTC NCC NAR YTG GCC CT

N=A+G+C+T; R=A+G; W=A=T; Y=C+T.

5.2.9.1. PCR components and cycling conditions

The PCR reactions were performed in a 50 μL reaction containing 2 μM of each primer and 1.5 mM $MgCl_2$ buffer. All other components were as described in section 5.2.5.3. One hundred nanograms of HSV-1, PRV, VZV and EHV-4 viral DNA or plasmid were used as template for the specific primers. Samples were denatured for 5 minutes at 94°C prior to thermal cycling. The samples were then subjected to 60 cycles of amplification by denaturing the reactions for 30 seconds at 94°C followed by annealing at 55°C for 1 minutes. A 7 minutes extension at 72°C was performed prior to cooling of the samples to 6°C.

In order to assess the effect of an increase in annealing temperature on the reaction specificity the above reaction was performed with the exception that annealing was performed at 65°C. The results under both conditions were compared.

5.2.9.2. Results

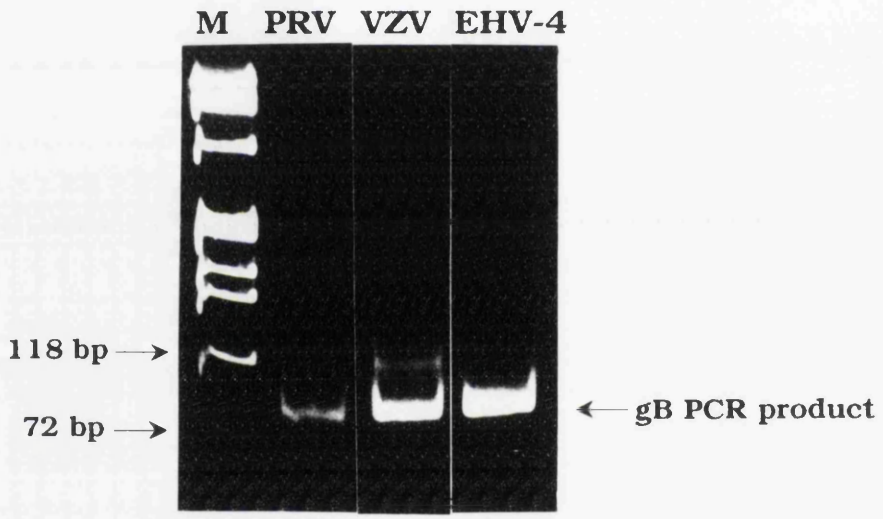
Following electrophoresis and ethidium bromide-staining (section 2.5.6.) amplified PCR product from each of the viral templates was detectable. On comparison of the 65°C and 55°C annealing temperatures, primer-dimer formation appeared to be significantly reduced using the 65°C annealing temperature although overall signal was comparable. Results obtained from three of the viral templates following cycling at 55°C are shown in Fig 5.4.

In conclusion although the 65°C annealing temperature appeared to reduce primer dimer formation, it was decided to err on the side of caution and continue with a 55°C annealing temperature which is less stringent.

Fig 5.4. Ethidium bromide-stained PCR products following PCR amplification of viral templates PRV, VZV and EHV-4.

M= ϕX 174/Hae III marker.

Fig 5.4. Amplification of various viral templates



PCR product of the expected size is detectable from each of the viral templates.

5.2.10. EXPERIMENT 7
Comparison of 22-mer and 23-mer 3' primers

To further streamline the reaction and enable the use of a 96-well microtitre format the decision was made to reduce the 3' primer by 1 base from a 23-mer to a 22-mer. By removing the terminal base all possible codon usage for the pentapeptide would still be covered and yet only 16 3' primers would be required. To test whether this reduced assay sensitivity, a comparison of an EHV-4 specific 23-mer (Table V.VIII.) and a new 22-mer was made.

5.2.10.1. PCR components and cycling conditions

An EHV-4 template was used in the reaction with 10-fold dilutions of the DNA from 1 μg to 1 ng. Primers were used at a concentration of 2 μM with all other reaction components as described previously (section 5.2.5.3.). Cycling conditions with a 55°C annealing step, described in Table V.VII.(experiment 5A) were used.

5.2.10.2. Results and conclusions

Amplified PCR product was clearly detectable in all dilutions of template DNA used. The signal obtained on ethidium bromide-stained gels was comparable using the 3' 22-mer or 23-mer. The results obtained from the 10 ng and 1 ng dilutions using the 22-mer and 23-mer primers are shown in Fig 5.5.

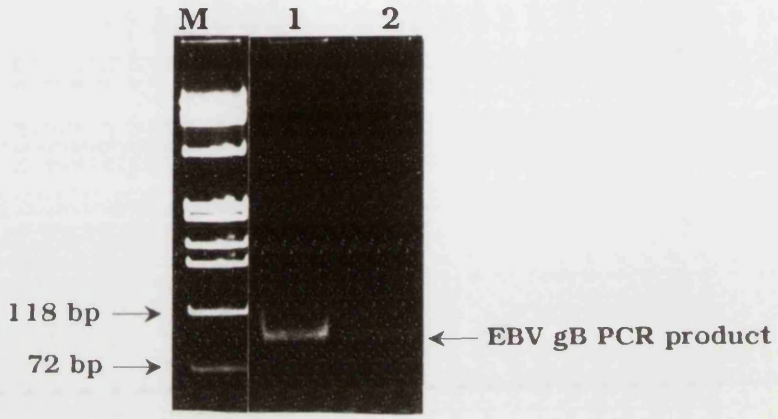
Therefore in the manufacturing of the final primer set all 3' primers were 22 bases in length. This reduction in primer number allows the reaction to be performed in a 96-well microtitre format. This enables easier handling of the assay and also helps reduce overall cost.

Fig 5.5. Comparison of 22-mer and 23-mer 3' primers. Ethidium bromide-stained PCR products following PCR amplification as described in section 5.2.10.

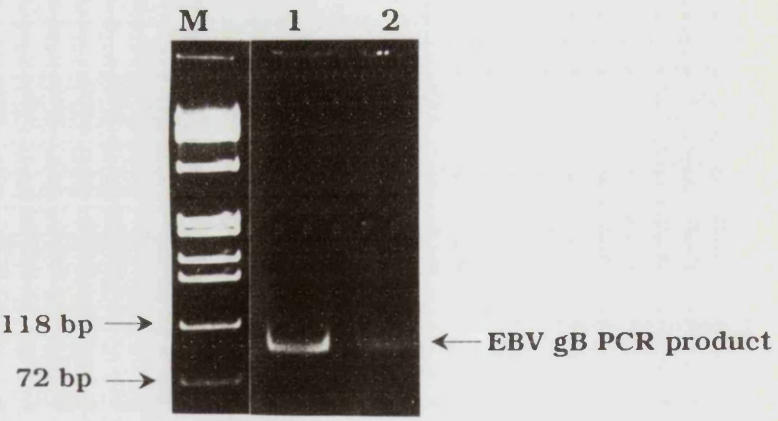
M= ϕX 174/Hae III marker; 1= 10 ng EHV-4 DNA; 2= 1 ng EHV-4 DNA.

Fig 5.5. Comparison of 22-mer and 23-mer 3' primers

A 22-mer



B 23-mer



Results obtained using the 22-mer and 23-mer are comparable.

5.2.11. EXPERIMENT 8
Improved cycling conditions for the detection of viral sequences

A complete set of primers was manufactured for the final assay with six, 128-fold degenerate 5' primers (Table V.IX.) and sixteen, 64-fold degenerate 3' primers (Table V.X.). Prior to utilising the primer combinations for the analysis of clinical DNA samples, further cycling conditions were compared in an attempt to enhance both specificity and sensitivity of the reaction. The different primers used in the assay have a wide range of melting temperatures and therefore thermal cycling conditions are critical as discussed in section 5.2.3. From previous experiments, a high initial annealing followed by a reduction in temperature did not appear to increase overall product yield (sections 5.2.5.5. and 5.2.8.3.). A further optimisation experiment to test the effect of a low initial annealing step using known viral templates was performed.

Table V.IX. 128-fold degenerate 5' primers, all 23-mers

conserved amino-acid sequences

Primer	Hind III	C	Y	S/N/T	R	P
233	TCA AAG CTT	TGY	TAY	WNN	CGA	CC
234					CGG	CC
235					CGT	CC
236					CGC	CC
237					AGA	CC
238					AGG	CC

Table V.X. 64-fold degenerate 3' primers, all 22-mers

conserved amino acid sequences

Primer	Eco R1	G	L	Q	G	E/Y/P/Q/L
201	ATC GAA TTC	NCC	NAR	YTG	ACC	T
202					GCC	T
203					TCC	T
204					CCC	T
205					ACC	C
206					GCC	C
207					TCC	C
208					CCC	C
209					ACC	A
210					GCC	A
211					TCC	A
212					CCC	A
213					ACC	G
214					GCC	G
215					TCC	G
216					CCC	C

N=A+G+C+T; W=A+T; Y=C+T.

5.2.11.1. PCR components and cycling conditions

One hundred nanograms and ten ng of both PRV and VZV DNA were used as template. Primers 206/236 and 206/235 were used at a concentration of 2 μM for the PRV and VZV templates respectively. A Hot Start PCR was performed for each sample with all other reaction components as in section 2.5.4.3.

Samples were denatured for 5 minutes at 94°C followed by 60 cycles of amplification. In the initial 3 cycles, reactions were denatured at 94°C for 30 seconds then annealed at 37°C for 1 minute followed by an increase in temperature to 72°C over a 2 minute period. Fifty seven further rounds of amplification were then performed as specified in Table V.XI.

Table V.XI. Thermal cycling conditions of Experiment 8

Experiment No	Temp 1	At temp	Temp 2	At temp	Cycles
8A	94°C	30 sec	65°C	1 min	x57
8B	94°C	30 sec	55°C	1min	x57

5.2.11.2. Results and conclusions

In experiment 8A with cycling at 65°C the gB gene product signal was lost in both the 100 ng and 10 ng dilutions of the VZV template. Only non-specific banding was observed with no visible product of the expected size on ethidium bromide-stained gels. Specific PCR product of the correct size was detectable in the 100 ng and 10 ng dilutions of the PRV template although many non-specific bands were also visible following gel electrophoresis.

In experiment 8B with 55°C annealing, PCR product was visible on ethidium bromide-stained gels in both the 100 ng and 10 ng dilutions of VZV and PRV templates. The presence of non-specific product was not evident in either of the samples.

From these experiments it would appear that the lower annealing temperature of 37°C has allowed annealing of the primers to both target and non-target sequences, however annealing at 65°C in later cycles was possibly too stringent for further amplification of specific PCR product. In the second experiment with an annealing temperature of 55°C only specific product was detectable and therefore these cycling conditions were selected for use on DNA from a clinical sample.

5.2.12. EXPERIMENT 9
Detection of HHV-6 and EBV in a DNA sample from a case of AILD

In order to test whether the optimised conditions described in section 5.2.11.2. would be sufficient for the detection of herpesvirus sequences within a DNA sample from a clinical case, the following experiment was performed.

5.2.12.1. PCR components and thermal cycling conditions

One hundred nanograms of DNA from a case of angioimmunoblastic lymphadenopathy (AILD) previously shown to contain HHV-6 type B and EBV by Southern blot analysis were used as template in each reaction. Detection of HHV-6 in this sample required a long exposure of the Southern blot to autoradiographic film. EBV was barely detectable on Southern blot analysis and was in fact initially scored as negative when a Bam HI-C probe specific for EBV was used. A Bam HI-W probe, from the internal repeat sequence of EBV, was required to detect the virus. PCR was performed in a 50 μL volume containing 2 μM of each primer. Hot Start PCR was performed (section 5.2.5.2.) and all other reaction components were as stated in section 5.2.5.3. All possible primer combinations were used in a 96 well format as shown in Table V.XI.

Reactions were denatured in a Perkin-Elmer 9600 machine for 5 minutes at 94°C. Samples were amplified under the optimised conditions described in section 5.2.10.2. Products of the PCR reaction were electrophoresed and ethidium bromide-stained (section 2.5.6.).

Table V.XII. Primer combinations in a 96-well microtitre plate format

5' primers 233-238 were added to 3' primers 201-216 in the combinations shown below.

233	234	235	236	237	238	233	234	235	236	237	238
201	201	201	201	201	201	209	209	209	209	209	209
202	202	202	202	202	202	210	210	210	210	210	210
203	203	203	203	203	203	211	211	211	211	211	211
204	204	204	204	204	204	212	212	212	212	212	212
205	205	205	205	205	205	213	213	213	213	213	213
206	206	206	206	206	206	214	214	214	214	214	214
207	207	207	207	207	207	215	215	215	215	215	215
208	208	208	208	208	208	216	216	216	216	216	216

5.2.12.2. Results and conclusions

A PCR product of the predicted size was detectable on an ethidium bromide-stained gel from reactions containing primer pairs 209/234 and 212/234 (Fig 5.6.A). The gel was electroblotted and hybridised to an HHV-6-specific gB probe (sections 2.5.7. and 2.4.4. respectively) derived from sequences internal to the primers and this confirmed that the sequences were HHV-6-specific (Fig 5.6.B). A gel containing PCR products from the primer set 207/236, which should have amplified EBV-specific product, was also blotted and hybridised to an EBV-specific internal probe. Although PCR product of the expected size was not visible on the ethidium bromide-stained gel, specific EBV gB product was clearly detected following hybridisation.

The product of the PCR reaction using primers 209/234 was cloned (section 2.6.) and 4 clones were sequenced commercially (Alta-Bioscience). The sequence data confirmed the authenticity of the product, although primer usage was different from that expected from the published sequences of HHV-6.

This experiment clearly demonstrates that this assay has the capability of detecting herpesvirus sequences present in DNA from a clinical

Fig 5.6. Results obtained from a clinical DNA sample following PCR amplification as described in section 5.2.12. Primers used are detailed in the figure.

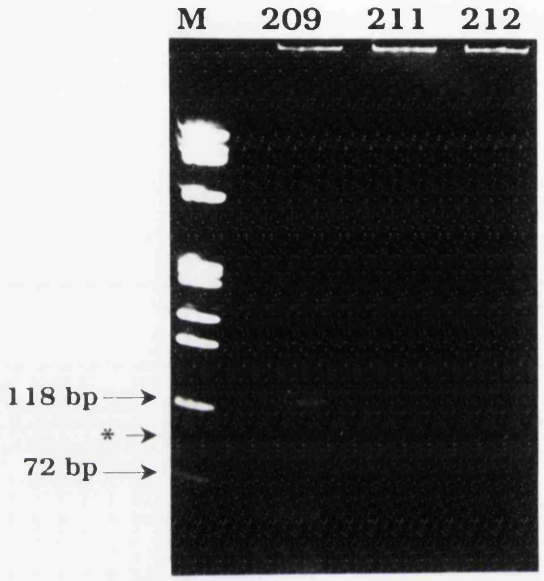
Fig **A** shows ethidium bromide-stained PCR products. Fig **B** shows the result obtained following hybridisation with an HHV-6 probe.

HHV-6 probe sequence: 5 'GAC GTT TAG CTT TGT GAA CTC CAC GCC TGA 3'.

Fig 5.6. Detection of HHV-6 in a clinical DNA sample

A Ethidium bromide-stained gel.

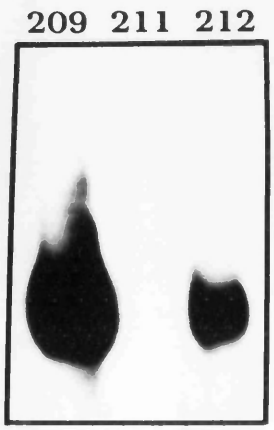
3' primers as shown below. 5' primer 234.



* indicates HHV-6 gB gene PCR product

B Hybridisation with an HHV-6 specific probe.

3' primers as shown below. 5' primer 234.



sample. The sensitivity of the assay is highlighted by the fact that HHV-6 was detected in this sample by Southern blot analysis only following a long exposure of the autoradiograph, however HHV-6 specific gB product was clearly demonstrable on an ethidium bromide-stained gel.

5.2.13. EXPERIMENT 10
Comparison of TaqStart and Ampliwax Hot Start PCR

As described in section 5.2.4.2. Ampliwax beads can be used to facilitate a Hot Start for the PCR reaction. Hot Start has been shown to improve the sensitivity of PCR when low copy number of target is present. The Hot Start is therefore of critical importance in an assay such as this where low viral copy number may be present in a non-specific background. The Ampliwax beads have a disadvantage in that they are cumbersome to handle and this may lead to an increased chance of contamination. Furthermore in this assay where there are 96 reactions the use of the Ampliwax beads can be time consuming. Recently an antibody to Taq polymerase (TaqStart, Clontech) has been made available for use in Hot Start PCR. The anti-Taq antibody binds to the enzyme rendering it inactive until the initial denaturing step of the reaction. In this experiment a comparison was made between Ampliwax and TaqStart antibody.

5.2.13.1. PCR components and thermal cycling conditions

DNA from the HHV-6 type A infected J-Jhan cell line was used as template in each of these experiments. HHV-6 was an ideal template for the reaction, as the calculated *Tms* of the primers required for amplification of this template were among the lowest in the overall primer set. If the annealing temperature was too high for efficient annealing of the HHV-6 primers, then the assay would be inefficient.

A PCR reaction was performed in a 96-well format as described in section 5.2.12.1. One hundred nanograms of DNA from J-Jhan cells infected with HHV-6 were used as template in each reaction. Ampliwax beads were used in the initial experiment to facilitate a Hot Start and

cycled at 55°C as described in section 5.2.11.1. In the following experiment TaqStart, at a ratio of 28:1 antibody to enzyme, was used following the manufacturers instructions and under identical reaction conditions as used with the Ampliwax. A further comparison was made using a ratio of 20:1 TaqStart to Amplitaq. Following thermal cycling products of the PCR reactions were assessed as described previously (section 2.5.6.). In addition products were subjected to dot-blotting as described below.

5.2.13.2. Dot-blotting of PCR products onto nylon membrane

To streamline screening of PCR products with specific gB probes, products were denatured and subjected to dot-blotting onto nylon membrane (Amersham) using a 96-well dot blot manifold (Scotlab) following the manufacturers instructions. The membrane-bound products were hybridised to an end-labelled HHV-6-specific internal probe (section 2.4.3.) and washed under the appropriate conditions (section 2.4.4.). Comparisons of the electrophoresed ethidium bromide-stained products obtained and autoradiographs of hybridised dot blots were made.

5.2.13.3. Results

On ethidium bromide-stained gels PCR product was detectable in reactions containing primers 209/234, 211/234 and 212/234. Following hybridisation an HHV-6-specific oligonucleotide probe signal was clearly demonstrable in each of the above reactions. The typical results obtained for both Ampliwax and TaqStart reactions are shown in Fig 5.7.

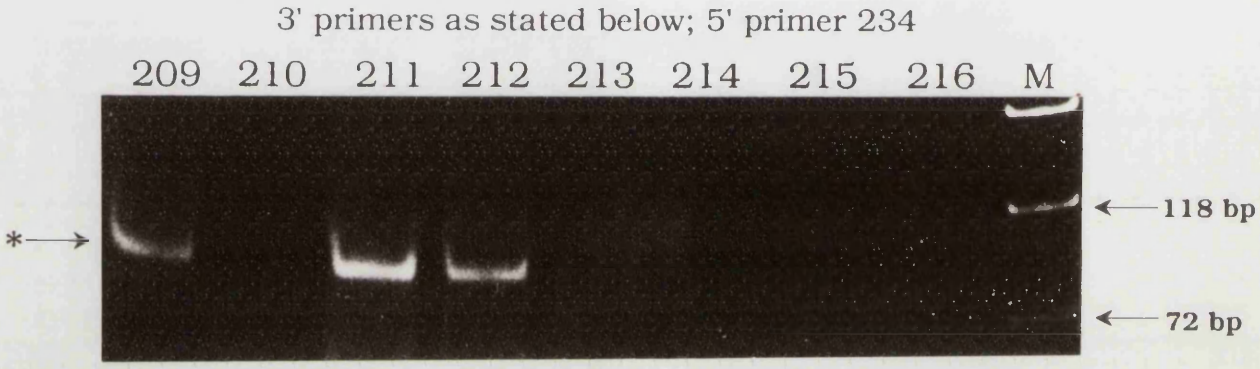
Comparisons of autoradiographs from Ampliwax and TaqStart experiments were made. Hybridisation signal obtained was comparable for each of the experiments with Ampliwax and TaqStart. The manufacturers recommend a 28:1 ratio of antibody to Amplitaq enzyme although suggest that titrations may be required. The results obtained

Fig 5.7. Detection of HHV-6 in an HHV-6 type A infected cell line (J-Jhan). Fig **A** shows ethidium bromide-stained PCR products following amplification as described in section 5.2.13.1. Fig B shows specific gB product following hybridisation with an HHV-6 probe. Results shown were obtained using Ampliwax beads. Experiments using TaqStart antibody were comparable to that shown.

HHV-6 probe sequence: 5 'GAC GTT TAG CTT TGT GAA CTC CAC GCC TGA 3'.

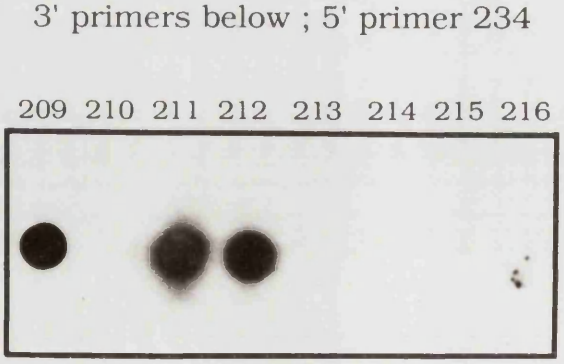
Fig 5.7. Detection of HHV-6 type A

A Ethidium bromide-stained gel of PCR products



* indicates the positive result obtained with primers 209/234, 211/234 and 212/234

B Hybridisation of dot-blot with an HHV-6 specific probe



HHV-6 specific product detected with primers 209/234, 211/234 and 212/234

showed that a 20:1 ratio was satisfactory for our purposes and this was chosen as a standard for further experiments.

In conclusion therefore the TaqStart product would appear to be a suitable substitute for Ampliwax beads within a Hot Start PCR. The use of TaqStart has greatly improved the handling and throughput of the assay.

5.2.14. METAPHOR AGAROSE GEL ELECTROPHORESIS

Until this point, PCR products were electrophoresed on 8% polyacrylamide gels as described in section 2.5.6. The complete assay necessitated the use of 12 gels. Aside from being time consuming, the handling of large amounts of acrylamide is potentially hazardous. Metaphor agarose is an intermediate melting temperature agarose with twice the resolution capabilities of the finest sieving agarose. A 4.0% agarose gel, equivalent to an 8% acrylamide gel in sieving qualities, was poured. Products from the complete assay were electrophoresed on a single large gel (Anachem) at 150 V for 1.5 hour. The gel was stained and resolution compared to acrylamide gel electrophoresed products. The results were not only comparable with acrylamide, but bands were sharper with no "smiling" apparent at the edges of the gel.

The use of Metaphor agarose for the detection of PCR products following amplification with herpesvirus gB primers will greatly enhance the efficiency of the overall screening process.

5.3. DISCUSSION

This study was designed to aid in the further investigation of known or as yet uncharacterised herpesvirus sequences that may be present in affected lesions of HD. The experimental data have shown that the assay has the capacity to detect herpesvirus gB sequences if 1 copy of the herpesvirus genome is present in 1% of cells within a tissue mass. This sensitivity has been achieved using ethidium bromide-stained gels for the detection of PCR product and not following hybridisation of product with specific probe. This is of paramount importance in a study such as this in which we wish to identify new or as yet uncharacterised sequences for which no specific probes are available.

The sensitivity of the assay was initially assessed utilising an EBV-positive DNA sample as template and further optimised with a range of herpesvirus templates. Numerous comparative experiments were performed for the optimisation of both reaction and thermal cycling conditions. The assay has been validated by the detection of two different herpesvirus genomes within a clinical DNA sample previously investigated for the presence of theses viruses by Southern blot analysis. The herpesvirus gB sequences within this case were shown to be EBV and HHV-6 on the basis of preferential primer usage and confirmed by hybridisation with type specific internal oligonucleotide probes. The HHV-6 specific product was clearly visible following ethidium bromide-staining and hybridisation with an HHV-6 probe specific for the region internal to the primer sequences. The authenticity of the product was further confirmed by cloning and sequencing. HHV-6 had previously been demonstrated in this case following Southern blot analysis and hybridisation to the 9 kb HHV-6 probe pZVH14. Hybridisation to this probe gave rise to a weak signal following exposure to radiographic film. This emphasises the sensitivity of the PCR assay.

To enable the detection of a low copy number of herpesvirus sequences within template DNA, 60 cycles of PCR amplification were performed in this assay. A problem associated with increased cycle numbers within a PCR reaction is the increase of non-specific products. The use of degenerate primers may further augment this problem. Despite extensive experiments for the optimisation of thermal cycling conditions,

non-specific product was apparent using many primer sets. When known viral sequences are present in template DNA, PCR product is clearly detectable in the expected size range and therefore can be distinguished from non-specifically primed product. However, an experimental PCR system where sensitivity of the assay is maintained and a reduction in background priming is achieved would be advantageous. To achieve this goal a sensitive automated detection system such as that described in section 5.2.6.2. may enable a reduction in cycle numbers and therefore an eventual decrease in non-specific fragments within the PCR. This would allow more discrimination in the selection of PCR products for further investigations such as cloning and sequencing.

The recent purchase of an automated DNA sequencer (DNA 4000 sequencer, Licor) within the Department of Veterinary Pathology has enabled the use of 5' infra-red labelled primers for PCR amplification. The resulting infra-red labelled PCR products can be detected on the automatic sequencer using a laser scanner. This detection system employs similar methodology to the fluorescein-labelled detection system described previously. The sophisticated software will allow more accurate sizing of PCR products for selection and further characterisation. In addition, numerous samples can be analysed simultaneously and this will increase efficiency and throughput of the assay. Preliminary experiments suggest that the detection of PCR products using this technology is superior to the sensitivity found by conventional ethidium bromide-staining. This detection system may enable a reduction in cycle numbers while maintaining the necessary PCR reaction sensitivity. The presence of non-specific product may therefore be reduced or eradicated utilising the automated system. Further experiments are required to assess these predictions.

An additional advantage of an automated system for PCR product analysis, is that it will enable the use of 7-deaza-2' deoxyguanosine (c^7dGTP) within the PCR. Compton (1990), used c^7dGTP in their PCR reactions combined in a dGTP mixture, to combat problems associated with GC rich regions of target DNA. McConologue *et al.*, (1988) have demonstrated that the use of c^7dGTP is vastly superior in PCR reactions with difficult templates and allows increased specificity. When designing

our primers we did not include c^7dGTP, because although the overall efficiency of reactions is not affected, intercalation of ethidium bromide is hindered and results in less efficient detection of amplified DNA. The use of an automated gel system may dispense with the need for ethidium bromide staining of PCR products and therefore the inclusion of c^7dGTP in the assay may enhance sensitivity and specificity in future experiments. More recently Weiss *et al.*, (1994), have described the use of silver staining for the detection of reactions containing c^7dGTP. In contrast to ethidium bromide-staining, the binding of silver ions is not impaired by c^7dGTP. This is therefore another detection method which merits investigation, when c^7dGTP is substituted for conventional dGTP within our PCR reactions.

The nested analysis discussed in section 5.2.6.3. will allow small DNA samples from HD biopsy specimens to be investigated for the presence of herpesvirus sequences.

At present this assay has been shown to have the sensitivity required to investigate clinical DNA samples for the presence of herpesvirus gB sequences. Utilising this assay HD samples can be screened for the already characterised herpesviruses by using type specific internal gB probes. Any visible PCR product of the expected size, that does not hybridise with the known probes can be cloned for sequencing and further investigation. This assay may lead to the identification of a different herpesvirus in the non EBV-associated cases of HD.

CHAPTER 6
GENERAL DISCUSSION

The aetiology of HD is not known. Viruses are thought to play a key role, however in common with other malignant diseases it is likely that other factors e.g., oncogene activation are also involved. EBV has been detected in around 40% of HD cases (section 1.4.1.). EBV LMP-1 is expressed by the RS cells in EBV-positive cases. The paediatric and older age groups of HD are frequently EBV-associated. In contrast, the young adult group particulary of the NSHD subtype have not been associated with EBV (section 1.4.1.1.).

At the start of this work it had been shown that the t(14;18) translocation was present in tumours from HD cases (Chapter 3). LMP-1 has been shown to upregulate Bcl-2 protein expression, and therefore the potential induction of *bcl-2* by the t(14;18) translocation or by LMP-1 in EBV-associated cases suggested that increased Bcl-2 expression might be playing a role in the pathogenesis of HD (section 1.4.4.1.). Results obtained from our study indicate that the t(14;18) translocation is an infrequent finding in HD. In addition no correlation between LMP-1 positivity and Bcl-2 expresssion was noted. Other studies have corroborated these findings as discussed in section 3.3. The t(14;18) translocation detected in HD tumour tissue may have been present in the reactive component of these tumours. This suggestion is certainly feasible as Bcl-2 expression can be detected in normal lymph node tissue (section 1.6.). In a further study LMP-1 expression appeared to be independent of both CD23 and Bcl-2 expression in EBV-positive HD tumour samples (Armstrong *et al.*, 1992a). In conclusion the t(14;18) translocation is infrequently found in association with HD and Bcl-2 expression would appear not to be a critical event in the pathogenesis of the disease. The exact role of LMP-1 in the pathogenesis of the EBV-associated cases of HD disease remains unclear.

An animal model for the propagation of the malignant cells of HD would be advantageous for subsequent investigation and characterisation of the RS cell. The SCID mouse model has been shown to be a useful tool in the investigation of a number of human malignancies (section 1.6.). The results obtained from our study showed that, although the propagation of NHL tissue was readily supported in the SCID environment, growth of the malignant cells from HD tumour tissue was unsuccessful. Tumours obtained in the SCID model following injection

of HD tumour tissue resembled EBV-driven lymphoproliferations as observed in studies following engraftment of PBMCs from EBV-seropositive donors. Relatively few studies have attempted the growth of HD tumours in SCID mice and therefore further studies such as that detailed in Chapter 4, are needed to arrive at definitive conclusions on the use of such a strategy for HD research. At present, however, the SCID model would not appear to be a suitable system for the propagation of HD-derived tumour material. The problems observed in our study may be due to the lack of the functioning immune system in the SCID mouse as cell-cell interactions may play an important role in the pathogenesis of HD.

The difficulty in the growth of HD tumour tissue *in vivo*, as described above, and *in vitro* has impaired both the immunophenotypic and genotypic analysis of the disease. As previously discussed, the relative paucity of malignant cells present within the tumour mass has impaired the characterisation and investigation of the RS cell (section 1.2.4.). PCR is a sensitive technique widely used for the detection of DNA sequences. This can be applied to single cells at both the DNA and RNA level. The advent of sensitive techniques such as single cell PCR analysis has enabled more specific studies to be performed. The technique has been used for the study of IgH gene rearrangements in the RS cells of HD cases (Kuppers *et al.*, 1994). Studies like this enable characterisation of the RS cell away from the reactive component of the tumour mass. This technique has also been used to demonstrate p53 mutations within the RS cells of HD tumour specimens and suggests that the p53 gene may play an aetiological role in the disease (section 1.2.6.). Further studies are required to clarify the significance of this finding however the use of single cell PCR for the investigation of RS cells in HD may prove to be invaluable.

In a recent study of HD tumour tissue quantitative PCR analysis has revealed an association between the t(2;5) translocation and the disease (Orscheschek *et al.*, 1995). This translocation is often found in T-cell derived anaplastic large cell lymphoma which morphologically and immunohistochemically resembles HD (Agnarsson & Kadin, 1988). The translocation results in a fusion product with tyrosine kinase activity which may regulate cell growth and be involved in tumorigenesis. The

authors suggest that t(2;5) may be used as a clonal marker in a proportion of HD cases.

The young adult peak observed in HD, in particular the HDNS subtype is non EBV-associated. Epidemiological studies suggest that this group of cases may have an infectious aetiology (section 1.2.5.). In order to investigate HD tumour tissue for the presence of herpesvirus sequences, in particular the non EBV-associated cases, a PCR strategy was devised. As discussed in Chapter 5 this assay has the sensitivity to detect herpesvirus sequences present in RS cells. In addition the assay has the capability of distinguishing different herpesviruses. This is of particular importance in HD tumour tissue where different herpesviruses may be present in both the RS cell and/or the reactive component of the tumour.

The primers for this assay were based on two well conserved penatapeptide regions of herpesvirus gB genes. The gB sequence of an additional herpesvirus, the canine herpesvirus, was recently published (Limbach *et al.*, 1994). On examination of the amino acid sequence of the canine gB gene the conserved regions, from which the primers of our assay are based, were present. This highlights the highly conserved nature of these sequences in gB genes across the herpesvirus family, and confirms the potential use of this assay for the detection of new or as yet uncharacterised herpesvirus sequences.

Section 5.3. discussed the possible use of an automated screening system for the detection of PCR derived products utilising infra-red-labelled primers. Preliminary experiments suggest that the overall sensitivity of this methodology is superior to ethidium bromide-staining. This may enhance detection of viral sequences at low copy number and possibly decrease the amount of template DNA required. Samples enriched for RS cells, or pooled single RS cells used as template in this reaction, may reveal a new or as yet uncharacterised herpesvirus in the non-EBV associated cases of HD.

The studies described above emphasise that PCR is a useful tool in the investigation of tumour tissue. Many different applications have been described utilising PCR technology however most noteworthy in this

context is the discovery of a new hantavirus, which is the suspected aetiological agent of the newly identified respiratory disease syndrome (Hjelle *et al.*, 1994). The virus was isolated using primers designed to conserved regions of previously characterised hantaviruses and highlights the use of PCR technologies based on conserved regions of known genes for the identification of novel agents.

The herpesvirus gB assay will be used alongside other molecular techniques for the detection of unknown viral sequences. One such technique known as representational difference analysis has been described where differences between two genomes can be isolated and cloned utilising a PCR based methodology (Lisitsyn *et al.*, 1993). Preliminary results obtained within our laboratory suggest that this is a promising technique for the isolation of viral sequences (personal communication, J. MacKenzie). Recently this technique has enabled the identification of herpesvirus-like DNA sequences from patients with Kaposi's sarcoma, the most common neoplasm occurring in persons with AIDS (Chang *et al.*, 1994). The study concludes that the herpesvirus sequences detected may prove to be a new herpesvirus although the exact relationship between virus and disease has yet to be clarified. The possible discovery of a new herpesvirus supports the notion that as yet uncharacterised herpesviruses exist and may be associated with other malignancies.

In conclusion, the exact nature of the RS cell in HD remains an enigma and the role of viruses within the disease is not clear. However, the advances made in recent years in HD studies and the advent of sophisticated technology for subsequent investigations, may ultimately answer the unresolved issues.

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