The Involvement of oncogenic DNA viruses in Hodgkin's disease

by

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DECLARATION

I declare the work reported in this thesis is my own original work except where otherwise stated and has not been submitted for any other degree.

SUMMARY

At the outset of this project Epstein-Barr virus (EBV) was associated with a proportion of cases of Hodgkin's disease (HD). Clonal EBV genomes were detected in HD tumour material however the localisation of EBV within the malignant cells had not been clearly demonstrated. Following the availability of monoclonal antibodies to the EBV latent genes, the expression of LMP-1 and EBNA-2 was investigated in a series of HD cases. EBV LMP-1 was expressed in Reed-Sternberg (RS) cells in the majority of cases studied, however there was a lack of expression of EBNA-2. The detection of LMP-1, which has been shown to have oncogenic potential, has strengthened the evidence that EBV is involved in the pathogenesis of a proportion of HD cases. These results indicated that a distinct pattern of EBV latent gene expression, designated Lat II, was observed in HD.

As LMP-1 has been shown to upregulate a number of cellular genes, the expression of CD23 and bcl-2 in HD were examined. CD23 and bcl-2 were rarely detected in RS cells. There was no correlation between LMP-1 expression and the presence of CD23 or bcl-2. These results indicate that the role of EBV in HD is independent of the upregulation of CD23 and bcl-2.

Further evidence that EBV is localised to RS cells in HD was obtained following the detection of EBER RNA in RS cells using an *in situ* hybridisation technique. Comparison of techniques to detect EBV in HD tumour material indicated that the EBER RNA *in situ* hybridisation assay was the most useful and reliable method of determining EBV latent infection. We have categorised HD cases which were EBV-positive by *in situ* assays or using Southern blot hybridisation for the detection of clonal EBV genomes as *EBV-associated.*

Using the above criteria the epidemiological features of HD were investigated with respect to EBV. Paediatric HD cases, in particular cases <10 years of age, older adults and cases of MCHD subtype were strongly EBV-associated. There was no evidence that EBV is a useful prognostic marker.

Although the epidemiological features of HD suggest that young adult HD is most likely to be caused by an infectious agent our results indicated that these cases, in particular NSHD, were seldom EBV-positive. We have speculated that another virus may be involved in this age group. In order to eliminate the possibility that other known DNA viruses may be involved in HD, we examined clinical samples from HD, NHL and reactive conditions for the presence of adenovirus, SV40, LPV and HHV-7. We have not detected any of the viruses in these clinical samples. The implications of these results will be discussed further in this thesis.

ABBREVIATIONS

ABC-AP	avidin-biotin complex linked to alkaline phosphatase
AIDS	Acquired immune deficiency syndrome
ALL	Acute lymphoblastic leukaemia
APAAP	alkaline phosphatase anti-alkaline phosphatase
AP	alkaline phosphatase
ATL	Adult T-cell leukaemia
BL	Burkitt's lymphoma
CI	confidence interval
DEPC	diethyl pyrocarbonate
DNA	deoxyribonucleic acid
eBL	endemic Burkitt's lymphoma
EBER	Epstein-Barr encoded RNA
EBNA	Epstein-Barr nuclear antigen
EBV	Epstein-Barr virus
FBS	foetal bovine serum
HBLV	Human B-lymphotropic virus
HBSS	Hank's balanced salts solution
HBV	Hepatitis B virus
HCC	hepatocellular carcinoma
HCMV	Human cytomegalovirus
HCV	Hepatitis C virus
HD	Hodgkin's disease
HHV-6	Human herpesvirus-6
HHV-7	Human herpesvirus-7
HIV	Human immunodeficiency virus
HLA	Human leucocyte antigen
HPV	Human papilloma virus
HSV	Herpes simplex virus
HTLV	Human T-lymphotropic virus
Ig	Immunoglobulin
IM	infectious mononucleosis
LCL	lymphoblastoid cell lines
LMP	latent membrane protein
LPHD	lymphocyte predominant HD

LDHD	lymphocyte depleted HD
LPV	lymphotropic papovavirus
MCHD	mixed cellularity HD
MHC	major histocompatibility complex
NHL	non-Hodgkin's lymphoma
NPC	nasopharyngeal carcinoma
NSHD	nodular sclerosis HD
OD	optical density
OR	odds ratios
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
pmol	picomoles
PP	paralytic poliomyelitis
PRV	Pseudo rabies virus
Rb	retinoblastoma
RNA	ribonucleic acid
rpm	revolutions per minute
RS	Reed-Sternberg
sBL	sporadic Burkitt's lymphoma
SV40	Simian virus 40
TBS	tris buffered saline
TCR	T-cell receptor
TEMED	N,N,N',N'-tetramethylethyldiamine
TSP	Tropical spastic paraparesis
μg	micrograms
μL	microlitres
UV	ultra violet
VZV	Varicella-zoster virus

Chapter 1

General Introduction

1.1 Aim of study

A number of malignant diseases are known or suspected to have an infectious aetiology. For many years it has been suggested that the malignant lymphoma Hodgkin's disease (HD) may be caused by a virus. Recently a proportion of HD cases have been associated with the lymphotropic herpesvirus Epstein-Barr virus (EBV). The role of EBV in the pathogenesis of HD is unclear and at the initiation of the work presented in this thesis few studies had investigated the localisation of EBV within the affected tissues in HD. This study examined the localisation of EBV within Reed-Sternberg (RS) cells and the reactive infiltrate.

In order to gain some insight into the role EBV plays in HD, the presence of the B-cell activation antigen, CD23 and proto-oncogene bcl-2 was investigated in RS cells. These cellular genes have been shown to be upregulated by EBV, *in vitro*. The EBV status of HD cases was further correlated with the epidemiological features of the disease. A range of HD cases and non-Hodgkin's lymphomas (NHL) were also analysed for the presence of other oncogenic DNA viruses.

1.2 Classification of lymphomas

Classification systems for malignant lymphomas are complicated (Lukes & Collins, 1975, 1977, Gerard-Marchant *et al.*, 1974, Rosenberg, 1982, Bennett *et al.*, 1991). New disease categories have now been recognised as more detailed information concerning lymphomas becomes available (Harris *et al.*, 1994). The Kiel classification system of non-Hodgkin's lymphoma (NHL), which was recently updated, subdivided lymphomas into low and high grade classes of either B- or T-cell origin (Stansfeld *et al.*, 1988). Categorisation of lymphomas based on

immunophenotyping as a single entity is not ideal as the normal counterpart of many neoplastic cells has not been identified. Harris *et al.* (1994) has suggested that other features such as treatment category and clinical aggressiveness of NHL should be included in classification systems. In contrast to the NHL classification system the subdivision of HD using the Rye classification system has remained stable and is generally accepted as pathologically relevant (Bennett *et al.*, 1991, see section 1.3.3).

1.3 Hodgkin's disease

Hodgkin's disease (HD), an unusual disease of lymph nodes, was first described by Thomas Hodgkin in 1832 (Hodgkin, 1832). It was not until the latter half of the last century that Sternberg and Reed described the large bilobed or multinucleated giant cells, which are now termed Reed-Sternberg (RS) cells (Sternberg, 1898, Reed, 1902). Early investigators suggested that HD was a form of Mycobacterium tuberculosis (Sternberg, 1898), or was a chronic immunological disorder. The malignant nature of HD has now been established (Kaplan, 1980, 1981, Rosenberg, 1989).

1.3.1 Clinical features of HD

The clinical features of HD include lymphadenopathy; the most common sites of presentation are in the neck and mediastinal lymph nodes (Mauch, 1993). In addition constitutional B symptoms i.e. pyrexia, night sweats or weight loss have been observed (Selby & McElwain, 1987).

1.3.2 Histopathological classification and definition of HD

The histopathological features of HD have been examined by Lukes and Butler (1966). The diagnosis of HD is dependent on the identification of Reed-Sternberg (RS) cells or a morphological variant of this cell, within an appropriate histological background (Lukes, 1971). The cellular infiltrate is heterogeneous in HD consisting of lymphocytes, plasma cells, histiocytes, eosinophils and polymorphonuclear leukocytes. The diagnosis of HD requires the presence of RS cells within the context of this cellular background as RS-like cells have been identified in other conditions including infectious mononucleosis and NHL (Strum *et al.*, 1970, Tindle *et al.*, 1972).

The most commonly used classification system of HD, proposed at an International meeting in Rye, New York (Lukes *et al.*, 1966) simplified the Lukes and Butler system as shown in Table 1.1. The Rye classification system incorporates the proportion of RS cells and the nature of the cellular infiltrate within a lesion and this system forms the basis for the division of HD into four subgroups; nodular sclerosis (NSHD), mixed cellularity (MCHD), lymphocyte depleted (LDHD) and lymphocyte predominant (LPHD) (Lukes *et al.*, 1966, Butler, 1992). These four histological subtypes differ in their histopathological appearance (reviewed by Krajewski & Jarrett, 1994), age and sex distribution (McKinney *et al.*, 1989, Alexander *et al.*, 1989, 1991a) and prognosis (reviewed by Ultmann, 1992). These pathological features are summarised in the following sections 1.3.2.1-1.3.2.4.

Table 1.1 Histological classification systems of HD

Classification system		
Lukes & Butler[1]	**Rye[2]**	**Harris[3]**
Lymphocytic & histiocytic		
Nodular	Lymphocyte predominance	Lymphocyte predominance
Diffuse		Lymphocyte-rich classical
Nodular sclerosis	Nodular sclerosis	Nodular sclerosis
Mixed cellularity	Mixed cellularity	Mixed cellularity
Diffuse fibrosis	Lymphocyte depletion	Lymphocyte depletion
Reticular		

1 Lukes & Butler (1966) The pathology and nomenclature of Hodgkin's disease. Cancer Res, 26:1063-1081
2 Lukes *et al.* (1966) Report of the nomenclature committee. Cancer Res, 26:1311
3 Harris NL *et al.* (1994) A revised European-American classification of lymphoma neoplasms: a proposal from the International Lymphoma Study Group. Blood 84:1361-1392

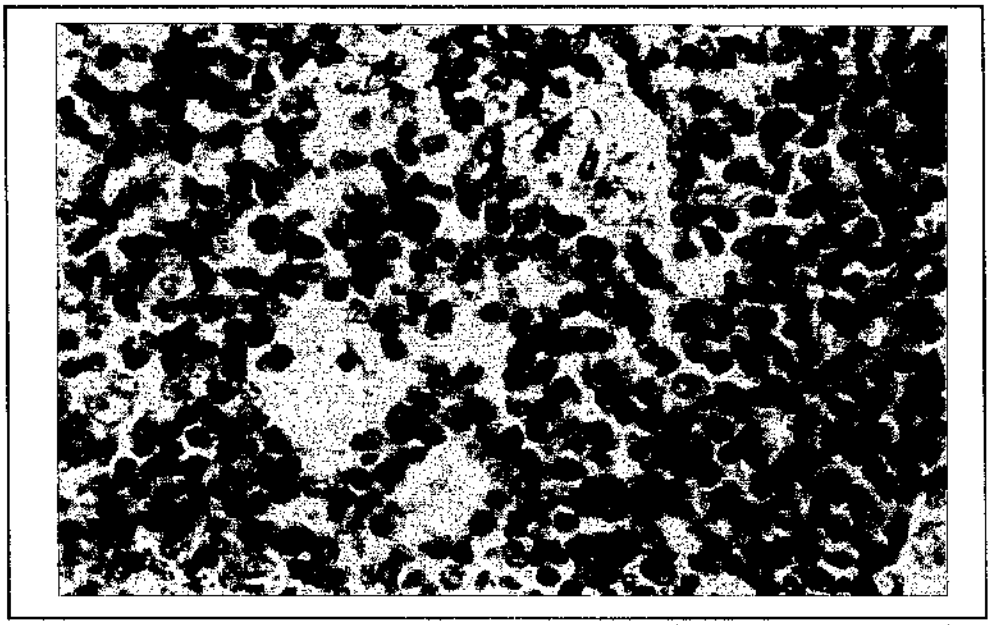
1.3.2.1 Lymphocyte predominance HD

Lymphocyte predominance HD (LPHD) involves between 5% and 10% of cases of HD and has been divided into nodular lymphocytic and histiocytic predominance (NLPHD) and diffuse lymphocytic and histiocytic predominance (DLPHD) types. This subtype of HD presents mainly in young adult (15-34 years of age) males. LPHD is characterised by the presence of the L & H type of RS cell, or "popcorn" cells, with very few classical RS cells (Wright, 1989, Butler, 1992, Figure 1.1). Cases of LPHD have a distinct phenotype $CD45^{+}$, $CD15^{-}$, cytoplasmic J chains and B-cell-associated antigens^{+} (CD19, CD20, CD22) suggesting that the L & H cells are of B-cell origin (Poppema *et al.*, 1979a, 1979b, Stein *et al.*, 1986, Timens *et al.*, 1986, Nicholas *et al.*, 1990). These cases tend to be described as a distinct clinical entity (Pinkus & Said, 1988, Nicholas *et al.*, 1990, Mason *et al.*, 1994).

1.3.2.2 Nodular sclerosis HD

Nodular sclerosis HD (NSHD) is the most common form of HD involving 70% of cases (Holman *et al.*, 1983). The majority of cases are adults less than 40 years of age. A variant form of the RS cell, the lacunar cell is seen in NSHD. The diagnosis of NSHD requires the presence of classical RS cells, lacunar cells and nodular formation with orderly bands of birefringent collagen that partially or entirely subdivides abnormal lymphoid tissue into isolated nodules (Bennett *et al.*, 1991, Figures 1.2a). NSHD has been further subdivided into good and poor prognostic categories, NS-1 and NS-2, respectively (MacLennan *et al.*, 1989, Figure 1.2.b).

Figure 1.1 Lymphocyte predominance HD
Haematoxylin & eosin-stained section

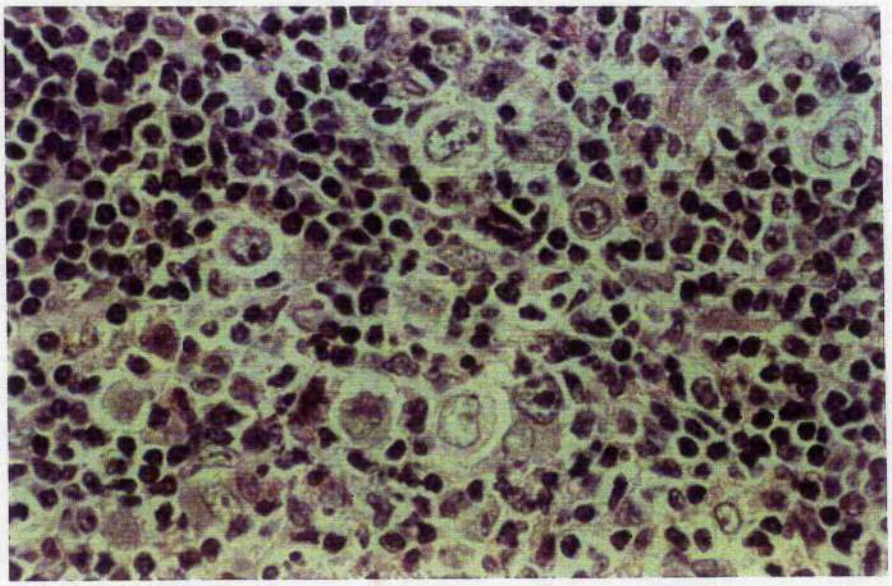


(Magnification x250)

Lymphocyte predominance HD case. L & H type of Reed-Sternberg cells are present in the section.

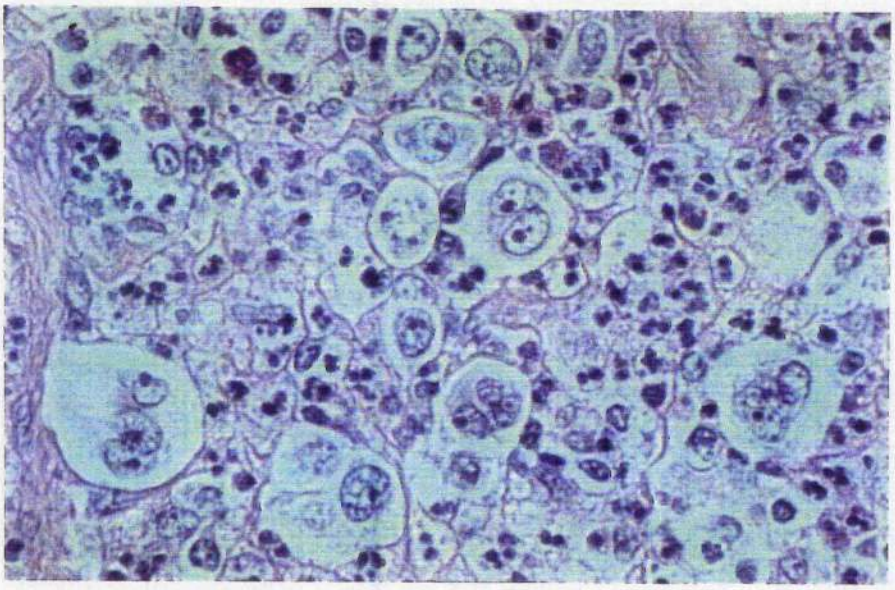
(kindly provided by Dr AS Krajewski, University of Edinburgh Medical School)

Figure 1.2a Nodular sclerosis HD
Haematoxylin & eosin-stained section



(Magnification x100)

Figure 1.2b Nodular sclerosis HD, NS-2
Haematoxylin & eosin-stained section



(Magnification x250)

(kindly provided by Dr AS Krajewski, University of Edinburgh Medical School)

1.3.2.3 Mixed cellularity HD

The mixed cellularity HD (MCHD) subtype accounts for between 20% and 40% of cases (Colby *et al.*, 1982). This subtype is more common in male patients and also within the older age category. Criteria for inclusion in this category are not always clear as cases which do not fulfil the morphological appearance of NSHD or LPHD tend to be classified as MCHD. RS cells are easily detected and the background cell population is mainly composed of small lymphocytes, plasma cells, neutrophils and eosinophils (see Figure 1.3).

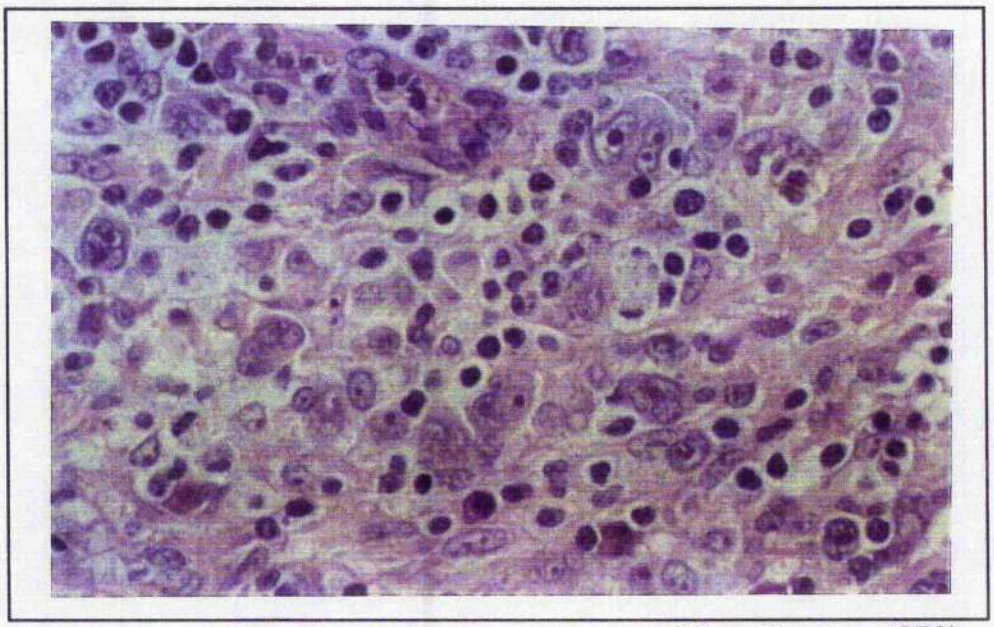
1.3.2.4 Lymphocyte depleted HD

Lymphocyte depleted HD (LDHD) is the least common type of HD and represents <5% of cases (Kant *et al.*, 1986). This subtype generally presents in the older age group (>49 years) and has been shown to be the most aggressive type of HD with the shortest median survival period. Two subtypes of LDHD are recognised, diffuse fibrosis and reticular (Lukes & Butler, 1966). On microscopical examination there is an overall cellular depleted appearance with disordered fibrosis and RS cells are present in almost every high power field.

1.3.3 Immunophenotyping and genotyping of HD cases

The cellular origin of the RS cell is still under debate (Hsu, 1990, Drexler *et al.*, 1992). The characterisation of RS cells has proven difficult due to the small number of malignant cells present in HD lesions. In addition studies to determine the phenotype of the cells have been limited due to the small numbers of lineage specific reagents available for use on routinely fixed tissue. Many different cell

Figure 1.3 Mixed cellularity HD
Haematoxylin & eosin-stained section



(Magnification x250)

Mixed cellularity HD case. Reed-Sternberg cells are present in the biopsy

(kindly provided by Dr AS Krajewski, University of Edinburgh Medical School)

types have been proposed as the normal counterparts of RS cells including B- and T-lymphocytes, macrophages, monocytes, myeloid cells and interdigitating reticulum cells (Drexler & Leber, 1988). Recent immunophenotyping studies on frozen and fixed HD tissue have been extensively reviewed by Drexler (1992).

Evidence for a lymphoid origin for RS cells is limited; variable percentages of positivities of the T-cell markers (CD2, CD3 or CD4) and B-cell markers (CD19, CD20 and CD22) have been found on the NSHD, MCHD and LDHD cases (Falini *et al.*, 1987, Casey *et al.*, 1989, Kadin *et al.*, 1988, Schmid *et al.*, 1991).

The classical histological subtypes, NSHD, MCHD and LDHD, showed expression of $CD15^{+/-}$, but there was a lack of CD45 expression on RS cells (Stein *et al.*, 1991). With the exception of LPHD, the majority of HD cases from the remaining histological subtypes expressed the activation markers, CD30 (Ki-1), CD25 (interleukin-2 receptor), CD74 (HLA-associated), HLA-DR and CD71 (transferrin receptor). These studies have enabled the distinction between MCHD and NHL, in particular peripheral T-cell lymphoma and T-cell rich and histiocyte rich B-cell lymphoma (Krajewski *et al.*, 1988, Harris, 1992).

A number of immunohistochemical studies have reported negative data which failed to support the notion that RS cells may have been derived from interdigitating reticulum or dendritic reticulum cells (Kadin, 1982, Carbone *et al.*, 1987, Kornstein *et al.*, 1986, Kennedy *et al.*, 1989). Curran and Jones (1977) implied that the origin of the RS cell may be follicular dendritic reticulum cells. In a more recent study, Delsol *et al.* (1993) reported expression of CD21 in a large proportion of HD cases. As CD21 is strongly expressed in follicular dendritic reticulum cells, it was suggested

that there may be a relationship between follicular dendritic reticulum cells and RS cells in HD.

Studies to investigate the presence of Immunoglobulin (Ig) and T-cell receptor (TCR) genes in HD have been used to determine the histogenesis of RS cells. These studies have been reviewed by Drexler (1992) and Diehl *et al.*, (1990). Rearrangement of Ig genes has been detected in a proportion of HD cases (Weiss *et al.*, 1986, Knowles *et al.*, 1986, Griesser *et al.*, 1987, Gledhill *et al.*, 1990). One report detected Ig gene rearrangements in 9 of the 35 HD cases studied (Gledhill *et al.*, 1991). A high incidence of TCRβ chain gene rearrangements was reported in two studies (Griesser *et al.*, 1987, Herbst *et al.*, 1989), however other studies have failed to confirm these findings (Schmid *et al.*, 1991, Gledhill *et al.*, 1990).

1.3.4 Epidemiology of HD

HD is a rare malignancy, representing only 1% of cancers diagnosed in the UK (Cartwright *et al.*, 1990). HD is uncommon in children of four years or younger (Kung, 1991).

In contrast to other malignancies, epidemiological studies have shown a bimodal age incidence curve for HD (MacMahon, 1966). This age incidence curve varies depending on the economic status of the countries studied. Correa & O'Conor (1971) described three epidemiological patterns of HD. In developing countries, a type I pattern is evident; the first age incidence peak occurs in childhood between the ages of 7 and 12 years and there is low incidence in young adulthood. The type III pattern is observed in developed countries. In this pattern the age incidence rises through childhood and the first peak is seen between the ages of 15 and 34

years. In both developed and developing countries, the incidence of HD increases in older age (Glaser & Swartz, 1990), however, in a recent study in the UK a plateau from 45 years of age was observed (McKinney *et al.*, 1989, Correa & O'Conor, 1971). An intermediate pattern of age incidence, designated type II, in which the childhood peak moves to a slightly older age, has been observed in countries undergoing socio-economic development and in rural areas of developed countries (Correa & O'Conor, 1971, Merk *et al.*, 1990, Alexander *et al.*, 1991b, Figure 1.4).

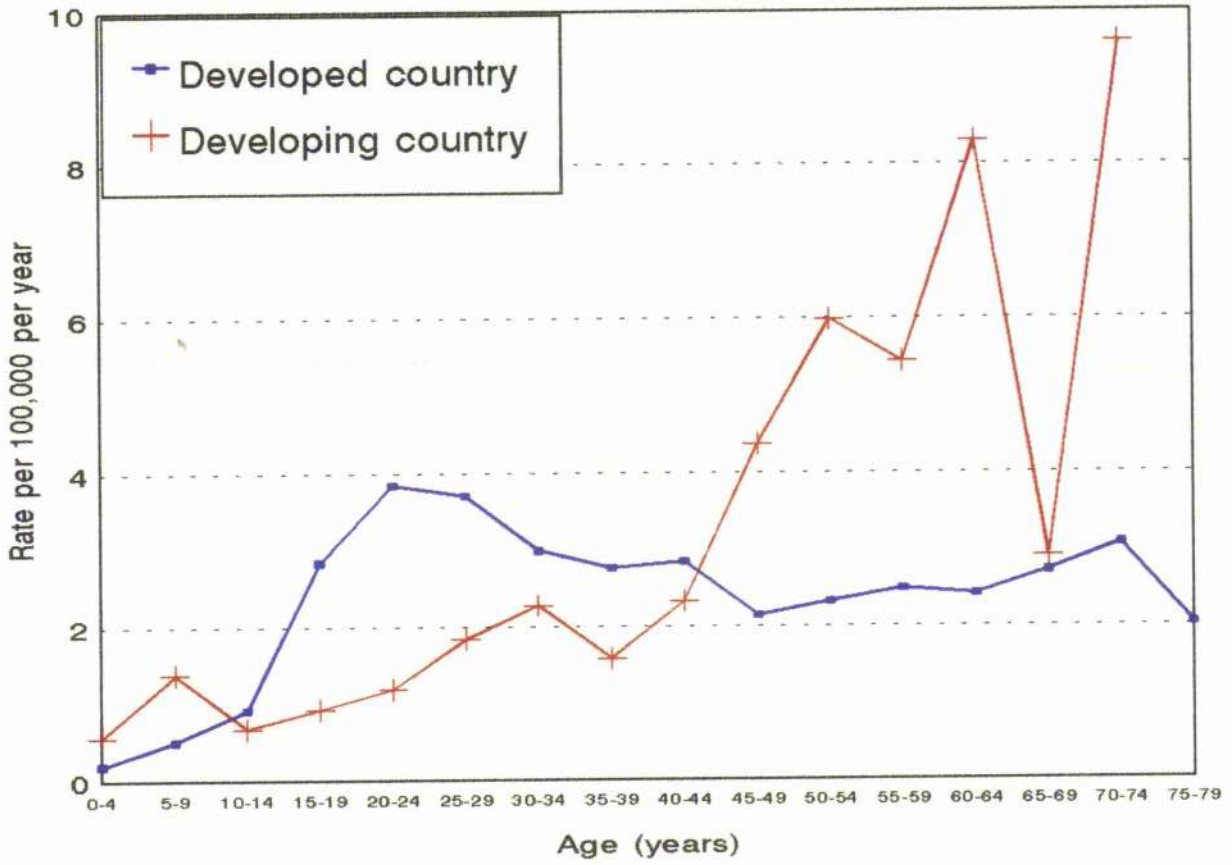
The proportion of the four histological subtypes of HD varies between age groups; NSHD predominates from the age of 11 years into young adulthood whereas MCHD is relatively more common in children under 10 years of age and in the older adult age group (McKinney *et al.*, 1989, Correa & O'Conor, 1971, Glaser & Swartz, 1990). In comparison with the NSHD subtype, when taken together, the other histological subtypes generally show a gradual increasing incidence with increasing age (Figure 1.5).

A high male:female ratio has been noted in children and is also present in older adults (Spitz *et al.*, 1986, McKinney *et al.*, 1989, Glaser & Swartz, 1990). In the young adult age group there are similar rates of female and male HD cases.

On the basis of the above epidemiological data, a number of hypotheses have been suggested to account for the aetiology of HD. MacMahon (1966) proposed that the three distinct age groups 0-14 years, 15-34 years and >49 years may have different aetiologies. He further suggested that HD in young adults may be caused by an infectious agent. Recent studies have supported the "two-disease" hypothesis in adult HD. The factors associated with the development of HD are different in young

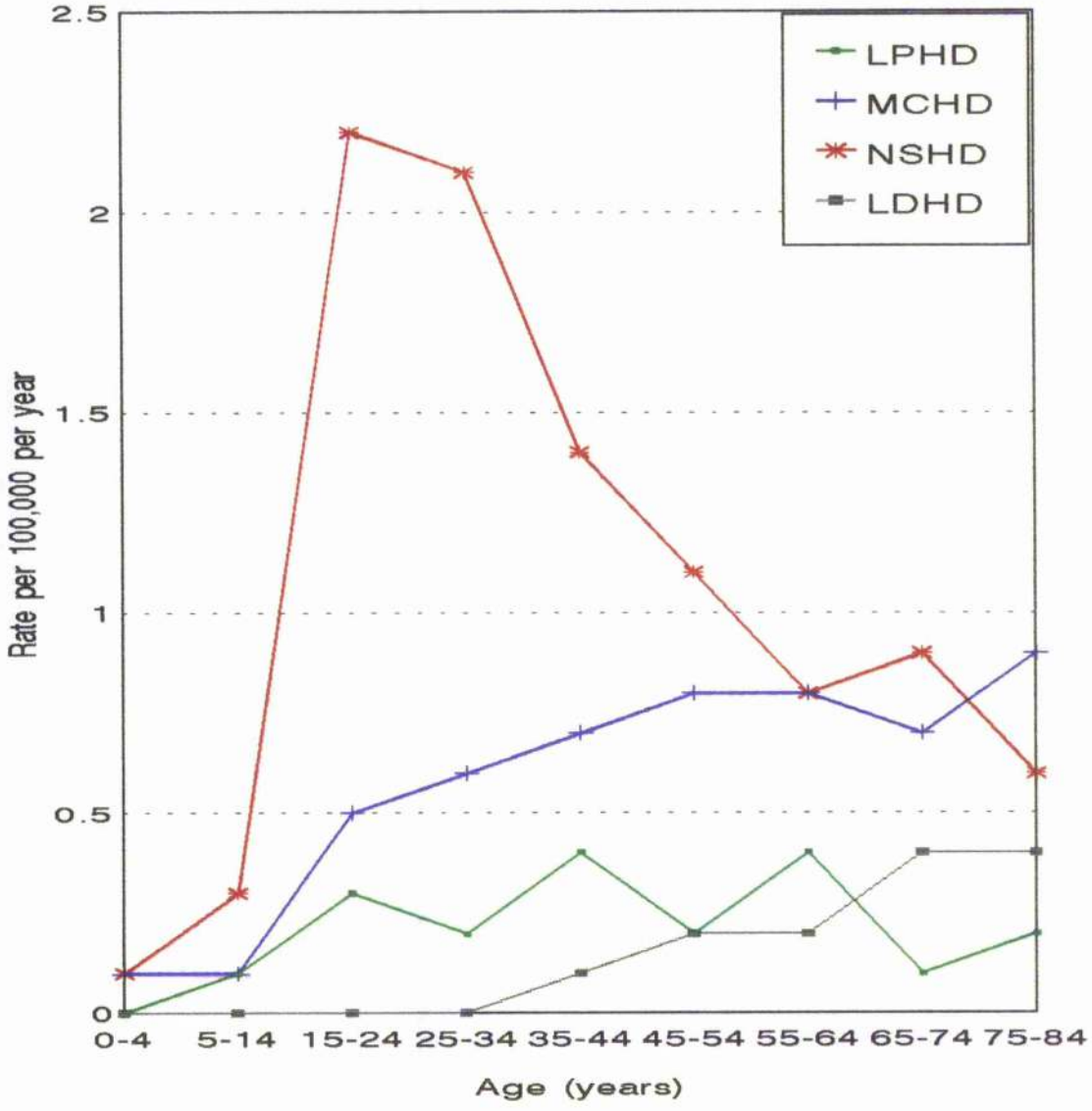
Figure 1.4 Age-specific incidence rates of Hodgkin's disease

Developed and developing countries



(reproduced from Cartwright RA, Alexander FE, McKinney PA, Ricketts RJ (1990) Hodgkin's disease. In Leukaemia and Lymphoma. An atlas of distribution within areas of England and Wales 1984-1988, London Leukaemia Research Fund, p.83 and Correa P, O'Conor GT (1971) Epidemiologic patterns of Hodgkin's disease. Int J Cancer 8:192-201)

Figure 1.5 Age-specific rates of Hodgkin's disease by histological subtype



(reproduced from McKinney PA, Alexander FE, Ricketts TJ, Williams J, Cartwright RA (1989) A specialist leukaemia/lymphoma registry in the UK. Part 1: incidence and geographical distribution of Hodgkin's disease. Int J Cancer 60:942-947)

adult HD cases from those observed in older cases (Cole *et al.*, 1968, Alexander *et al.*, 1991a).

The risk of developing HD in young adults has been associated with good living conditions during childhood, but this finding was not substantiated for older age HD cases (Gutensohn, 1982, Gutensohn & Cole, 1980, Paffenbarger *et al.*, 1977, Abramson *et al.*, 1978). Other epidemiological studies have indicated that high maternal education, small family size and early birth order children also increased the risk of developing HD (Gutensohn & Cole, 1981, Vianna & Polan, 1978). Abramson (1974) noted that the geographical distribution of HD was comparable to that of an infection which was subsequently controlled by vaccination - i.e. paralytic poliomyelitis (PP).

An analogy has been drawn between the epidemiological features of PP and HD - the *polio model* (Dauer, 1955, Gutensohn & Cole, 1977, Melnick, 1990). Prior to vaccination, environmental conditions determined the age at which infection with the polio virus was likely to occur and this subsequently determined the possible outcome of infection. In developing countries, exposure to common infectious agents was widespread in childhood and most individuals developed immunity before young adulthood. Children were infected early in life thus paralytic symptoms were observed in this age group. In developed countries infection in childhood was rare and therefore on exposure to the infectious agent older children and young adults were more likely to be infected. Infection in the latter age groups leads to a greater risk of developing paralytic manifestations of the infection.

HD in young adults, therefore, may be related to the age of exposure to infectious agents which in turn is determined by the socio-economic conditions encountered at

that time. In contrast to MacMahon's hypothesis, the polio model therefore predicts that the same infectious agent is involved in paediatric cases in developing countries and young adult cases in developed countries. These features are discussed further in Chapters 5 and 6.

1.4 Involvement of viruses in malignant disease

Both RNA and DNA viruses have been associated with the development of tumours in animals and less frequently in humans (Table 1.2). In particular the RNA virus family Retroviridae, subfamily Oncovirinae and the DNA virus families, Herpesviridae, Hepadnaviridae and Papovaviridae, which contains the subfamily Papillomavirinae, have all been associated with malignant disease in humans.

1.4.1 Herpesviruses

The family Herpesviridae represents a group of over 100 viruses characterised by the presence of a linear double stranded DNA genome ranging from 120 to 235 kilobase pairs (kbp) (Roizman & Baines, 1991). On examination by electron microscopy, the herpesvirions are enveloped particles of 120 to 300 nm in diameter (reviewed in Roizman & Furlong, 1974). Structurally, the virion consists of a core containing the linear double stranded DNA (Furlong *et al.*, 1972), an icosadeltahedral capsid of 100-120 nm in diameter containing the viral nucleic acid (Wildy & Watson, 1963), an amorphous tegument which surrounds the capsid (Roizman & Furlong, 1974) and an envelope from which viral glycoprotein spikes project from the surface.

Herpesviruses are classified according to the biological behaviour of the virus. The nature of the host range, cell tropism and site of latency are taken into consideration within the classification system (Roizman *et al.*, 1992). On the basis of these biological properties three subfamilies of herpesviruses, alpha, beta and gamma have been described.

Table 1.2 Viruses associated with tumours

Taxonomic group	Associated cancer
RNA viruses	
1. Retroviruses	
Oncoviruses	Hematopoietic cancers involving lymphoid, myeloid or erythroid cells, various sarcomas and carcinomas
2. Flaviviruses	Hepatocellular carcinoma
DNA viruses	
3. Hepadnaviruses	Hepatocellular carcinoma
4. Polyomaviruses	Various solid tumours
5. Papillomaviruses	Papillomas and carcinomas
6. Adenoviruses	Various solid tumours
7. Herpesviruses	Lymphomas and carcinomas
8. Poxviruses	Myxomas and fibromas

Reproduced from Benjamin T, Vogt PK (1990) Cell transformation by viruses. In: Virology, edited by Fields BN and Knipe DM, New York: Raven Press, p 381

The alphaherpesviruses have a wide host range, short replication cycle (18-20 hours) and establish latency in neuronal ganglia or lymphoid tissue. Members of this subfamily include herpes simplex virus-1 (HSV-1), herpes simplex virus-2 (HSV-2), varicella zoster virus (VZV), pseudorabies virus (PRV), equine herpesvirus-1 (EHV-1) and equine herpesvirus-4 (EHV-4).

In contrast the betaherpesviruses display a more restricted host range. Viral replication is slow and latency is observed in lymphoid cells, kidneys and salivary glands. Members of this subfamily include human cytomegalovirus (HCMV), and the recently identified human herpesviruses (HHV), HHV-6 and HHV-7, have also been assigned to this group.

Gammaherpesviruses have a limited host range *in vivo*. All members of this subfamily have been shown to replicate in lymphoblastoid cells *in vitro* and some may cause lytic infection in some types of epithelial and fibroblastoid cells. In addition, these viruses have the ability to immortalise either B-lymphocytes (γ1 subgroup) or T-lymphocytes (γ2 subgroup) and some can cause lymphoproliferative disease in the host. Latency usually occurs in lymphoid tissue. The γ1 subgroup of gammaherpesviruses includes EBV.

1.4.2 Herpesvirus life cycle

There are two distinct phases of the herpesvirus life cycle - lytic and latent phases. Primary infection by herpesviruses occurs in a number of stages in which the virus gains entry to the host cell and results in the release of progeny virus and death of the host cell. The virus also has the ability to establish a state of latency in the host. In order to initiate infection, the virus attaches through its envelope glycoproteins to receptors on the surface of the host cell. The receptor for EBV is the C3d (complement, CD21) receptor (Nemerow *et al.*, 1986). Following binding, penetration is complete in 1-2 hours (Fingeroth *et al.*, 1984). In the case of HSV-1 and HSV-2 the receptors are heparan sulphate proteoglycans (Wudunn & Spear, 1989). The virus penetrates by fusion of the viral envelope with the plasma membrane of the cell. Once the virus enters the cell, the capsids are released into the cytoplasm and are transported to the nuclear pores and the DNA-protein complex is released from the nucleocapsid into the nucleus. Upon release, the linear viral genome circularises by ligation of the terminal sequences. On entry to the nucleus, transcription and replication of viral DNA occurs (Honess & Roizman, 1974). Finally viral DNA and structural proteins are assembled into progeny virions. Both the core and capsid are assembled within the nucleus and the capsid is enveloped by budding through internal membranes (Roizman & Furlong, 1974). In the lytic cycle, host cell DNA and protein synthesis can be shut-off and this leads to host cell death (Wagner & Roizman, 1968).

The ability to establish latent infection is a characteristic of herpesviruses (Roizman, 1982). In latently infected cells, the viral genome is present as a circular or linear concatameric molecule (Rock & Fraser, 1985, Hurley & Thorley-Lawson, 1989). In the case of EBV, closed episomal forms have been detected (Lindahl *et al.*, 1976).

During this latent process only a limited number of viral genes are expressed. Latent gene expression of EBV is discussed in detail in sections 1.4.3.1-1.4.3.5. Herpesviruses can remain latent for long periods of time and thus avoid detection by the host immune system. Despite much study, the process of reactivation from latency remains elusive.

1.4.3 Epstein-Barr virus (EBV)

EBV was first isolated by Epstein *et al.* (1964) from cells cultured from Burkitt's lymphoma (BL) material. In the virion the EBV genome consists of a linear duplex DNA molecule of ~172 kbp (Baer *et al.*, 1984). This virus has been associated with a number of non-malignant and malignant diseases including infectious mononucleosis (IM), oral hairy leukoplakia, BL, nasopharyngeal carcinoma (NPC), NHL and lymphomas in immunosuppressed persons.

Primary infection with EBV usually occurs via the oral route in childhood. The virus is ubiquitous and ~90% of adults are infected (reviewed by Evans & Niederman, 1989). The virus shows a specific tropism for B-cells (Jondal & Klein, 1973, Greaves *et al.*, 1975). If infection is delayed until adolescence or young adulthood IM is observed in approximately half of the cases (Henle *et al.*, 1968). The clinical features of IM include lymphadenopathy, fever and pharyngitis all of which have been attributed to the host immune response to proliferating B-cells (Brown *et al.*, 1984).

Early studies suggested that EBV infected oropharyngeal epithelial cells in the first instance (Sixbey *et al.*, 1984, Wolf *et al.*, 1984). EBV was thought to spread from the oropharyngeal epithelium to B-cells (Allday & Crawford, 1988). Recent studies

have shown that patients with IM who were treated with acyclovir showed a reduction of EBV shedding from the oropharynx while the number of EBV-positive B-cells remained constant (Yao *et al.*, 1989a, 1989b). In addition treatment with irradiation and drugs following bone marrow transplantation resulted in the elimination of EBV from the patient through the destruction of haemapoietic tissue suggesting that this was an important site of EBV latency (Gratama *et al.*, 1988). From these data, Niedobitek and Young (1994) proposed that B-cells were important in primary infection and persistence of EBV.

EBV has the ability to transform human B-lymphocytes *in vitro* and thus produce lymphoblastoid cell lines (LCLs) (Pope *et al.*, 1968). It has been reported that only one particle of EBV is required to immortalise a B-lymphocyte (Henderson *et al.*, 1977). The viral genome is present in multiple episomal copies and has a restricted range of gene expression (reviewed by Speck & Strominger, 1989). The method of immortalisation of B-cells by EBV is not completely understood, however a number of gene products have been identified in LCL (reviewed by Kieff & Liebowitz, 1990). These latent genes include six EBV nuclear antigens, EBNA-1, EBNA-2, EBNA-3A,-3B,-3C and leader protein (LP), three membrane-associated proteins designated LMP-1, LMP-2A and LMP-2B and two small non-translated RNA molecules named EBV-encoded RNAs (EBER-1 and EBER-2).

Intensive research has been carried out to ascertain the role that these EBV latent genes play in the immortalisation process. To date, at least three of these viral genes have been shown to be required for immortalisation of B-cells: EBNA-1, EBNA-2, and LMP-1 (reviewed by Ring, 1994).

1.4.3.1 EBNA-1

The biological properties of the EBNA-1 protein have been identified. EBNA-1, a specific DNA binding protein, is required for the maintenance of the virus in episomal form through binding to the EBV plasmid origin of replication (*ori*P) (Yates *et al.*, 1984). The EBNA-1 gene is thus required to enable early proliferation of infected B-cells (Yates *et al.*, 1984, 1985). EBNA-1 is detected in all EBV-infected cells. A difference in promoter usage at different time points has been identified for different tumours. Expression of EBNA-1 in BL and in BL-derived cell lines appears to be driven from a promoter identified as Fp (Sample *et al.*, 1991, Schaeffer *et al.*, 1991). In LCL, Cp is the dominant promoter during transcription of all of the EBNA genes (Woisetschlaeger *et al.*, 1990). Recent studies of EBNA-1 expression in transgenic mice suggest that EBNA-1 is directly involved in malignant transformation (Wilson & Levine, 1992, J. Wilson, personal communication).

1.4.3.2 EBNA-2

Studies using the immortalisation-deficient mutant P3HR-1 (Hinuma *et al.*, 1967) in which the EBNA-2 gene and part of EBNA-LP is deleted (Rowe *et al.*, 1985), have shown that the EBNA-2 gene is required for immortalisation of B-lymphocytes *in vitro* (Cohen *et al.*, 1989, Hammerschmidt & Sugden, 1989). The reintroduction of the sequences deleted in P3HR-1, by homologous recombination, restored the ability to immortalise B-cells *in vitro*.

Two strains of EBV have been now been demonstrated: Types 1 and 2 or Types A and B, which differ in the EBNA-2, EBNA-3A, -3B, -3C gene loci (Adldinger *et al.*, 1985, Rowe *et al.*, 1989). These two antigenically distinct forms are represented by

B95/8 and AG876, respectively (Dambaugh *et al.*, 1984, Adldinger *et al.*, 1985, Rowe *et al.*, 1985, Zimber-Strobl *et al.*, 1986). Type 2-infected LCL *in vitro* show a poorer survival of individual cells, a lower growth rate and a lower saturation density than lines established with Type 1 virus (Rickinson *et al.*, 1987). EBV Type 1 virus has the ability to transform B-lymphocytes *in vitro* more readily than EBV Type 2 virus (Rickinson *et al.*, 1987). Following the reintroduction of EBNA-2 genes into the P3HR-1 genome, Cohen *et al.* (1989) showed that the differences in growth phenotype of LCL immortalised by Type 1 and Type 2 EBV strains are determined by the EBNA-2 type expressed.

Geographical differences between the two subtypes have indicated that EBV Type 1 is predominantly found in Western countries whereas Type 2 virus has been restricted to Papua New Guinea and Africa (Zimber-Strobl *et al.*, 1986, Young *et al.*, 1987). In a recent study from the US, EBV Type 2 virus was detected in the oropharyngeal epithelial cells of healthy persons (Sixbey *et al.*, 1991). EBV Type 2 virus has also been associated with endemic BL, NPC and to a lesser extent sporadic BL (Young *et al.*, 1987, Goldschmidts *et al.*, 1992).

The role of EBNA-2 in the immortalisation process is not fully understood. However, EBNA-2 has the ability to increase the expression of LMP-1 (Abbot *et al.*, 1990) and the B-cell activation antigens, CD21 and CD23 (Cordier *et al.*, 1990, Wang *et al.*, 1990). Both EBNA-2 and LMP-1 can lead to the induction of CD23 expression, either individually of co-operatively (Wang *et al.*, 1990). CD23 is detectable at high-levels in EBV immortalised B-lymphocytes (Kintner & Sugden, 1981, Thorley-Lawson *et al.*, 1985) and is also expressed after B-cell activation by interleukin-4 or mitogen stimulation (Defrance *et al.*, 1987). The induction of this B-cell activation molecule appears to be a critical event in the immortalisation of B-cells by EBV.

Only EBV-infected B-lymphocytes which express CD23 have the ability to produce immortalised cells lines (Thorley-Lawson & Mann, 1985). During the infection of EBV-negative B-lymphoma cell lines with the immortalising EBV strain, B95/8, high levels of CD23 are expressed, however when these experiments are repeated using the non-transforming EBV strain P3HR-1, CD23 antigen is not induced (Calender *et al.*, 1987). The role of CD23 in HD is discussed further in Chapter 3.

1.4.3.3 EBV latent membrane protein (LMP-1)

EBV LMP-1, a 62 kd membrane protein, is located in discrete patches in the plasma membranes of immortalised B-cells (Liebowitz *et al.*, 1986, Hennessy *et al.*, 1984, Mann *et al.*, 1985). Structurally, the EBV LMP-1 protein is thought to consist of a short hydrophilic amino terminus, six hydrophobic transmembrane domains and a long hydrophilic acidic carboxy terminus (Mann *et al.*, 1985). This structure shows similarity to ion channels and the β-adrenergic and rhodopsin receptors (Fennewald *et al.*, 1984). Single-gene transfer of LMP-1 to EBV-negative BL cell lines reproduces many of the altered growth effects seen in the latent EBV infection of primary B-lymphocytes; growth in tight clumps, increased villous projection and increased expression of activation molecules (Wang *et al.*, 1988). Kaye *et al.* (1994) have recently confirmed these early experiments and shown that LMP-1 is essential for EBV-mediated transformation of primary B-lymphocytes. LMP is the only virus encoded protein which has been shown to have transforming ability, in isolation. In transfection experiments, the LMP-1 gene can transform the rodent fibroblast cell line, Rat-1 and the transformed cells induce tumours in nude mice (Wang *et al.*, 1985). Deletion experiments suggest that all regions of LMP-1 are essential for transformation of Rat-1 fibroblasts (Moorthy & Thorley-Lawson, 1993).

A recent study indicated that EBNA-2 is required for the expression of LMP-1 in B-cells (Abbot *et al.*, 1990). However, EBNA-2 is not expressed in all situations in which LMP-1 is expressed (Fahraeus *et al.*, 1988, Pallesen *et al.*, 1991a). LMP-1 has the ability to induce a number of cellular genes including CD23 (Wang *et al.*, 1990), CD30, CD40, the cellular oncogene, bcl-2 (Henderson *et al.*, 1991) and the cellular adhesion molecules LFA-1, LFA-3 and ICAM-1.

LMP-1 protein expression has been detected by immunohistochemical analysis in 50% of cases of NPC (Young *et al.*, 1988). A role for LMP-1 in malignancies of epithelial origin has been supported by studies showing that LMP-1 can induce transformation in human keratinocytes (Fahraeus *et al.*, 1990) and the abnormally controlled differentiation of epithelial cells (Dawson *et al.*, 1990). The detection of LMP-1 in B-cell proliferations in transplant patients also indicates that LMP-1 may be involved in the pathogenesis of lymphoproliferative disease *in vivo*. EBV latent gene expression in HD is described in detail in Chapter 3, section 3.4.

Role of other EBV gene products in the immortalisation process

1.4.3.4 EBNA-3A,-3B,-3C

EBNA-3A,-3B,-3C are present in latently infected B-cells (Petti *et al.*, 1990), and there is homology in the amino acid sequence of all three latent genes between Type 1 and Type 2 virus (Sample *et al.*, 1990). The role of these EBNAs was investigated by Tomkinson & Kieff (1992) using mutant recombinant EBV generated by homologous recombination. EBNA-3A and EBNA-3C are required for immortalisation of B-cells, whereas EBNA-3B is not required (Tomkinson *et al.*, 1993). There is accumulating evidence to suggest that EBNA-3C is a trans-

activator of LMP-1 and the cellular genes, CD23, CD21 and vimentin (Wang *et al.*, 1990, Allday *et al.*, 1993).

1.4.3.5 EBV-encoded RNAs (EBERS)

The expression of two small, non-translated, RNA polymerase III transcribed RNAs, EBER-1 and EBER-2, was recognised by Jat & Arrand (1982). These RNAs are located in the nucleoplasm of EBV transformed lymphocytes (Howe & Steitz, 1986, Wu *et al.*, 1990) where they assemble into ribonucleoprotein particles that are complexed with the La autoantigen (Lerner *et al.*, 1981, Howe & Steitz, 1986). However, using high resolution confocal laser scanning microscopy there is clear evidence for the presence of both EBERs in the cytoplasm of Raji Burkitt's lymphoma cells and EBV-immortalised LCL (Schwemmle *et al.*, 1992).

In latently infected cells, the EBERs are actively transcribed and it is estimated that there are up to 5 x 10^6 copies per cell (Howe & Shu, 1989, Clemens, 1993). In a recent *in vitro* study, EBV mutants in which the EBER genes were deleted did not differ from the wild-type EBV in their ability to immortalise B-cells (Swaminathan *et al.*, 1991). These results suggest that the EBER genes are not essential in the immortalisation of B-cells by EBV.

Evidence for a possible function for these small RNAs is now emerging. There is a similarity between the cellular U6 small nuclear RNA and the EBER RNAs indicating that they may play a role in RNA processing (Glickman *et al.*, 1988). Recently, a cellular protein, EBER-associated protein (EAP), which has been shown to be the ribosomal protein L22, has been shown to bind to EBER-1 (Toczyski *et al.*, 1991, 1994). The significance of EBER-1 binding to the L22 protein on cellular growth

control has been raised following a report of a chromosomal translocation involving the gene for L22 in some forms of myelodysplasia (Nucifora *et al.*, 1993). The ability of the EBERs to bind L22 is reminiscent of other viral proteins e.g. SV40 large T antigen and adenovirus E1A, which interact with the anti-oncogenic products i.e. p53 or retinoblastoma (Rb) gene (Levine, 1990, Shenk & Flint, 1991). It has been suggested that these RNAs may play a role in cell transformation.

The abundance of these RNAs during latent infection makes them good targets for the detection of EBV using *in situ* hybridisation. The application of this technology to the detection of EBV in HD biopsies is discussed further in Chapter 4.

1.4.3.6 Patterns of EBV latent gene expression

In B-cells three distinct patterns of expression of the latent gene products have been described (Rowe *et al.*, 1992). A restricted pattern of expression is found in BL in which only the EBNA-1 gene is detectable (Rowe *et al.*, 1987). In contrast LCL display the full complement of latent genes. An intermediate pattern of latency has now been identified in which only EBNA-1, LMP-1 and LMP-2 are expressed. These patterns of latent gene expression have been designated as Lat I, Lat III and Lat II expression, respectively. The pattern of latent gene expression in various EBV-associated malignancies has been examined. In BL the latency pattern corresponds to Lat I and in lymphomas in immunosuppressed persons to Lat III. An intermediate pattern or Lat II expression pattern has been observed in NPC *in vivo*.

1.4.4 Human Herpesviruses-6 and -7

Human herpesvirus-6 (HHV-6) was first isolated from patients with AIDS, AIDS-related lymphomas and other lymphoproliferative diseases (Salahuddin *et al.*, 1986).

As the virus was shown to have B-cell tropism in cord blood lymphocytes it was initially named human B-lymphotropic virus (HBLV). Molecular analysis of the viral genome confirmed HBLV as a new herpesvirus (Josephs *et al.*, 1986). Following reports that the target cell population for HBLV in cord blood lymphocytes was $CD4^+$ T-lymphocytes rather than B-cells the virus was renamed HHV-6 (Lusso *et al.*, 1987, Tedder *et al.*, 1987).

The HHV-6 genome is a linear, dsDNA molecule of 162 kbp (Martin *et al.*, 1991). HHV-6 is most closely related to HCMV (Lawrence *et al.*, 1990). Two subtypes of HHV-6 have been identified by restriction site polymorphisms; HHV-6 variant A and HHV-6 variant B which have been shown to have distinct biological, immunological and molecular properties (Jarrett *et al.*, 1989, Schirmer *et al.*, 1991, Aubin *et al.*, 1991, Ablashi *et al.*, 1991, 1993).

HHV-6 is widespread within the general population (Briggs *et al.*, 1988). The virus has been shown to be the causative agent of the childhood illness exanthem subitum (Yamanishi *et al.*, 1988).

HHV-6 has the ability to transform NIH 3T3 cells and these cells have been shown to cause tumours in experimental animals (Razzaque, 1990). An association between HHV-6 and human malignancy has been proposed following detection of

the virus in some human malignancies (Josephs *et al.*, 1988, Jarrett *et al.*, 1988, Gompels *et al.*, 1992, Torelli *et al.*, 1991, 1992).

More recently, the isolation of a novel human herpesvirus from purified $CD4^+$ T-lymphocytes of a healthy individual was reported by Frenkel *et al.* (1990). This virus has been shown to be distinct from other human herpesviruses and has been designated human herpesvirus-7 (HHV-7). There is only limited sequence homology between the DNA from HHV-6, HCMV or HHV-7 (Berneman *et al.*, 1992). Serological studies have shown that HHV-7 is prevalent within the population and infects early in childhood (Wyatt *et al.*, 1991, Clark *et al.*, 1993).

1.4.5 Adenovirus

Adenoviruses are non-enveloped viruses with a double stranded DNA genome of between 30-35kbp in size (Green *et al.*, 1967). A number of serotypes of adenovirus have been identified and these are associated with a range of diseases in humans including acute respiratory disease in children (Brandt *et al.*, 1969), conjunctivitis (Bennett *et al.*, 1957) and gastrointestinal disease (Yolken *et al.*, 1982).

The adenoviruses have been classified into six groups, A-F, based on the oncogenic potential of the virus (Horwitz, 1990). All six subgroups have the ability to transform primary rat cells *in vitro* (Freeman *et al.*, 1967, Graham *et al.*, 1974). Subgroup A consists of types 12, 18 and 31 which have been shown to be highly oncogenic and cause tumours in animals (Trentin *et al.*, 1962), subgroup B includes types 3, 7, 11, 16, and 35 and which have been described as moderately oncogenic and type 5 belongs to subgroup C which are essentially non-oncogenic (Baum,

1984). The virus has the ability to remain latent in adenoidal tissue and also in B lymphocytes (Abken *et al.*, 1987).

1.4.5.1 Adenovirus genes involved in transformation

The E1 domain of adenovirus, encoding an intermediate early transcript, is divided into two regions, E1A and E1B. This region has been implicated in transformation by adenovirus (Sambrook *et al.*, 1974) and both E1A and E1B are required for oncogenesis (Byrd *et al.*, 1988). The E1A gene, E1B gene of adenovirus and the polyoma middle T antigen has been shown to be sufficient for transformation of primary rodent cells (Ruley, 1983, Gallimore *et al.*, 1985, Moran & Mathews, 1987). The E1A and E1B gene products co-operate in transformation; one function of E1A is to bind the Rb protein thus inactivating its function (Whyte *et al.*, 1988) and pushing cells into cycle (Howe & Bayley, 1992) whereas the 19 kd and 55 kd products of E1B have an anti-apoptotic function. Despite the above biological properties of adenovirus, there are no reports of an association between adenovirus infection and malignant disease in humans.

1.4.6 Papovavirus

The family Papovaviridae contains the subfamilies Polyomavirinae and Papillomavirinae. Members of the subfamily Polyomavirinae include the simian virus 40 (SV40), human polyomaviruses, BKV and JCV and lymphotropic papovavirus (LPV). The genomes of the polyomaviruses are dsDNA molecules of ~5 kbp. These viruses have the ability to cause tumours in species other than their natural hosts; SV40 virus causes tumours in newborn hamsters. The polyoma and SV40 large T antigens have the ability to immortalise cells in culture (Colby &

Shenk, 1982). Large T antigen binds the Rb protein (Larose *et al.*, 1991) and p53 thus exerting an anti-apoptotic effect and pushing the cells into cycle (Zhu *et al.*, 1991, Kierstead & Tevethia, 1993). Using serological techniques, the reactivity with antibodies against the polyomaviruses, SV40 and LPV has been identified in human serum following immunisation with poliovirus vaccines (Horváth, 1972, Shak & Nathanson, 1976, Brade *et al.*, 1980, Takemoto *et al.*, 1982).

1.5 Malignant diseases associated with EBV

EBV is associated with an ever growing range of malignant conditions, including BL, NPC, HD and NHL.

1.5.1 Burkitt's lymphoma

BL is a malignant lymphoma which is classified as a high grade lymphoma, small non-cleaved cell group (Rosenberg, 1982). There are two forms of BL distinguished by their geographical distribution - endemic or African BL (eBL) and non-endemic or sporadic BL (sBL). The peak incidence of eBL occurs in children, 6-8 years, however the peak incidence of sBL is in older children of 10-12 years in Africa and adults in other parts of the world. The incidence of eBL mimics the distribution of malarial infection. Both the chronic antigenic stimulation and immunosuppressive effect of malarial infection are thought to contribute to tumour development. The distribution of sporadic BL is unrelated to that of malaria.

The most characteristic feature of both forms of BL is a chromosomal translocation involving the c-myc locus (Shiramizu *et al.*, 1991). In the majority of cases the translocation juxtaposes the IgH gene on chromosome 14 with the c-myc locus. Other translocations involve the rearrangement of c-myc to immunoglobulin light chain (IgL) lambda or IgL chain (kappa) loci (Cory, 1986)

Cofactors associated with the development of tumours have been observed in BL. Serological and molecular studies have supported the role of EBV in eBL and to a lesser extent in sBL. There are increased antibody titres to EBV in African patients with BL compared to a control group (Henle *et al.*, 1969) and EBV DNA is detected

in 98% of tumour biopsies from these eBL patients (Nonoyama *et al.*, 1973). In sBL, EBV DNA was detected in 20% of cases studied (Ziegler, 1977).

1.5.2 Nasopharyngeal carcinoma

NPC, an epithelial tumour, is distributed world-wide, however there is a much higher prevalence in South East China (de-Thé *et al.*, 1989). Undifferentiated NPC is characterised histologically by malignant cells with indistinct cytoplasmic bands (Gaffey & Weiss, 1990).

The suggestion that NPC may have an infectious aetiology has been borne out by the detection of EBV in the majority of undifferentiated NPC cases (zur Hausen *et al.*, 1970, Klein, 1979). The EBV genomes in undifferentiated NPC are clonal, thus suggesting that EBV plays a direct role in the pathogenesis of the disease (Raab-Traub & Flynn, 1986).

As with BL, other factors are likely to be involved in the development of NPC. The geographical distribution of NPC and BL do not mirror the prevalence of EBV in the community. Both genetic and environmental factors have been suggested as additional factors (de-Thé *et al.*, 1989). Dietary factors such as nitrosamines in salted fish, which affect EBV latency, have been linked to an increased incidence of NPC (Yu *et al.*, 1986).

1.5.3 Lymphomas in immunosuppressed persons

The presence of B-cell lymphomas in immunosuppressed persons has been linked to EBV. The role of EBV in these malignancies is not entirely clear, however the

immunosuppressive nature of the host is likely to enable the EBV-infected B-cells to proliferate in an uncontrolled manner.

The association between EBV and post-transplant lymphoma has been documented (Purtilo *et al.*, 1981). The prevalence of post-transplant lymphoproliferative disease after renal or liver transplant ranges from 1% to 2.3%, respectively (Ho *et al.*, 1985, Hanto *et al.*, 1985). In heart-lung transplant patients this figure increases to between 5% and 9% (Cleary *et al.*, 1984). EBV has been detected in a substantial proportion of these post-transplant lymphomas (reviewed by Purtilo *et al.*, 1992).

Acquired immunodeficiency has been linked to the increased development of malignant lymphoma. Malignant lymphoma has been observed in an increasing number of cases following infection with human immunodeficiency virus (HIV). These tumours have been designated as acquired immunodeficiency disease-related NHLs (ARLs). Morphologically these tumours are high grade B-cell NHL of large cell or Burkitt (small non-cleaved cell) type (Hamilton-Dutoit *et al.*, 1991). EBV has been associated with a proportion of ARLs. EBV is present in almost all primary central nervous system ARLs (MacMahon *et al.*, 1991), in most systemic immunoblast rich/large cell ARLs, however in only 30-40% of BL type ARLs (Hamilton-Dutoit *et al.*, 1993a,1993b). The presence of EBV in the tumour cells correlated with the tumour morphology and primary site of the lymphoma (Hamilton-Dutoit *et al.*, 1993).

In sporadic high grade NHLs of B-cell origin, EBV has been detected in the tumour cells in 5% of cases. In peripheral T-cell lymphomas, EBV has been detected in a greater proportion of cases (10%) than in the B-cell type (4%) (G. Pallesen, personal communication). The significance of EBV in these lymphomas is not clear.

1.5.4 Hodgkin's disease

The association between an infectious agent and HD has been debated. Both serological and molecular techniques have identified EBV in HD cases. Initial serological studies showed that patients with HD have elevated levels of antibody to EBV compared with control cases (Levine *et al.*, 1971, Henderson *et al.*, 1973, Hesse *et al.*, 1977). These increased antibody titres were present in HD patients with a past history of IM (Evans & Gutensohn, 1984) and occurred prior to diagnosis of HD (Mueller *et al.*, 1989).

Early studies failed to detect EBV genomes in HD biopsies (Pagano *et al.*, 1973, Lindahl *et al.*, 1974). In 1987 Weiss *et al.* reported the detection of EBV genomes in HD tumour material in 4 out of 21 cases using Southern blot hybridisation (Weiss *et al.*, 1987a). Since that first report the proportion of EBV-positive HD cases has been shown to vary between 17-41% (Weiss *et al.*, 1987a, Anagnostopoulos *et al.*, 1989, Boiocchi *et al.*, 1989, Gledhill *et al.*, 1991, Jarrett *et al.*, 1991a).

Analysis of the terminal repeat region of the EBV genomes indicated that in the majority of cases examined a single infectious event had taken place (Weiss *et al.*, 1987a, Gledhill *et al.*, 1991, Anagnostopoulos *et al.*, 1989, Jarrett *et al.*, 1991a). These above results support the suggestion that, in a proportion of HD cases, the EBV had infected a single cell, which had clonally expanded.

At the start of this study two research groups had published data on the localisation of EBV within the malignant cells of HD, the Reed-Sternberg (RS) cells. DNA *in situ* hybridisation studies indicated that EBV genomes were localised to the RS cells in seven out of the ten cases tested (Anagnostopoulos *et al.*, 1989, Weiss *et al.*,

1989). However signal to noise ratios were not good and it was not possible to examine large numbers of cases, or archival material, using this technique.

Epidemiological features of HD have suggested an infectious origin for some groups of HD (see section 1.3.5). The association between EBV and age at diagnosis in HD has been investigated using Southern blot analysis (Jarrett *et al.*, 1991a). Paediatric and older age cases were shown to be more likely to be EBV positive than young adult HD cases. In addition MCHD cases are more likely to be EBV positive than NSHD cases (Weiss *et al.*, 1987a, Staal *et al.*, 1989, Boiocchi *et al.*, 1989, Gledhill *et al.*, 1991). The relationship between EBV status, age and histological subtype in HD is further investigated in Chapters 5 and 6 of this thesis.

1.6 Proving a relationship between a virus and cancer

Proving a relationship between a virus and cancer is difficult particularly where tumours in humans are concerned. It is only possible to try to get close to establishing a causal relationship. Prevention of the tumour by vaccination against the infectious agent provides proof that the virus is necessary for the tumour development but at the present time this is not a practical possibility in the investigation of most cancers.

Viruses can cause tumours by direct and indirect mechanisms, however the following discussion relates only to direct mechanisms of oncogenesis. Direct mechanisms have been described as systems in which part or all of the virus is present in the malignant cell at some stage during transformation. There are few examples of viruses which cause transformation by a single hit; one such example is polyoma virus infection of hamster cells where the titre of the infecting virus is directly related to the number of transformed foci.

In most cases in which viruses are involved in tumour development viral involvement is likely to be one step in a multi-step process. Thus other factors, either genetic or environmental, are likely to be involved in the development of tumours. In transplant patients, the presence of EBV-associated B-cell lymphoproliferative disease may be a rare example of a tumour which is directly associated with the virus alone. In these cases even monoclonal tumours, can recede following withdrawal of immunosuppressive treatment and initiation of anti-viral therapy (Starzl *et al.*, 1970).

In the early 1800s Koch proposed a set of criteria designed to prove that a micro-organism was involved in a given disease, as described in section 1.6.1-1.6.4. These criteria would appear to be too stringent to establish a relationship between a virus and cancer and would fail to recognise important pathogenetic associations (Hall & Lemoine, 1991). The methods used to demonstrate a link between a virus and a tumour are described in the following sections. Examples are given to show the use and the problems associated with each method.

1.6.1 Demonstration of virus or viral nucleic acid within tumour cells

Strong evidence that the virus may be involved in tumour development is provided by the demonstration of the virus in all cells of a tumour. Tumours caused by a hit and run mechanism would not fulfil this criterion. One example of the latter mechanism of tumourigenesis is exhibited by bovine papillomavirus (BPV)-4. Bovine cells infected with BPV-4 cause metastatic tumours when transplanted into immunocompromised mice (Campo *et al.*, 1985, Gaukroger *et al.*, 1993). Viral nucleic acid cannot be detected within these tumours thereby providing evidence for a hit and run method of transformation in an experimental system. Due to the difficulty in proving a hit and run mechanism it is not clear whether this could be an important way in which viruses operate.

Viruses can be detected within tumours by electron microscopy or by demonstration of viral nucleic acid or protein. Viral particles are usually detected by electron microscopy after culture of tumour cells in the laboratory. Some tumours, such as cervical cancers associated with HPV-16, contain defective viral genomes and are unable to produce viral particles. At present, molecular analyses such as PCR, Southern blotting and *in situ* hybridisation are frequently used to detect viral

genomes within affected tissues. Expression of viral proteins within tumour cells provides evidence of viral infection and may help to explain the role of the virus in disease pathogenesis. The demonstration of expression of viral genes or proteins, particularly those known to have oncogenic potential, in tumour cells provides good evidence that the virus is contributing to tumour development.

1.6.2 Serological studies

Serological studies provide important clues to the association between a virus and cancer. In the case of HTLV-1, the distribution of adult T-cell leukaemia (ATL) and tropical spastic paraparesis (TSP), both of which are known to be causally associated with the virus, follows the distribution of the virus as determined by serological studies (Robert-Guroff *et al.*, 1982, Gessain *et al.*, 1985). In contrast to control cases almost all cases of ATL and TSP are HTLV-1 seropositive (Hinuma *et al.*, 1982, Clark *et al.*, 1985). Serological studies therefore played an important role in establishing the relationship between HTLV-1 and these very different diseases. The association between hepatocellular carcinoma (HCC) and Hepatitis B virus (HBV) has been demonstrated using serological techniques (Beasley *et al.*, 1981, 1982).

Serological studies of EBV are more complex since most adults are EBV seropositive, however in eBL EBV titres tend to be raised suggesting an association between the virus and the lymphoma (Henle *et al.*, 1969, de-Thé *et al.*, 1978). The situation regarding EBV titres in HD is complicated and not fully resolved.

Several tumours associated with viruses show a restricted geographical distribution, e.g. NPC. In these cases the distribution of cases appears to follow the distribution of co-factors and not that of the virus (see sections 1.5.1 and 1.5.2).

1.6.3 Transformation by viruses *in vitro*

The ability to demonstrate transformation by a virus *in vitro* provides supportive evidence for the role of a virus in oncogenesis, however in many cases it has not proven possible to propagate tumour viruses in cell culture. This may either be due to the lack of an appropriate system to culture the target population or to the presence of defective viruses. Conversely, genomes or parts of genomes from several viruses have been shown to have transforming properties *in vitro* but have not been associated with any naturally occurring tumours (reviewed by MacNab, 1987).

1.6.4 Tumour induction in experimental animals

The restricted host range of some viruses e.g. HTLV-1, EBV, HBV, has presented difficulties in establishing animal models In circumstances where it is possible to produce viral infection e.g. HTLV-1 infection of rabbits (Miyoshi, 1992), it has not been possible to reproduce the human disease.

These examples highlight the difficulty in laying down criteria for demonstrating an association between a virus and cancer. Current efforts should attempt to establish close links between virus and particular malignancies. Viruses may provide a single hit, or one of several hits, which ultimately lead to malignant transformation. Additionally, viruses may provide a single hit resulting in pre-malignant change and

a greatly increased risk of tumour development. Therefore any evidence showing the presence or expression of viral genes at any stage in tumour development may provide important evidence for an association between a virus and cancer.

1.6.5 Viruses and Hodgkin's disease

There are two difficulties associated with the demonstration of an association between viruses and HD. First, RS cells in HD are rare, representing <1% of the tumour population and therefore any method used to demonstrate the presence of virus must be both sensitive and, in the case of common viruses, permit cellular localisation of the virus to the tumour cells. Methods of localising EBV to RS cells are described in detail in Chapters 3 and 4.

Secondly, epidemiological evidence suggests that viruses linked to HD are likely to be common viruses which infect many individuals at a young age (see Chapters 5 and 6). EBV is associated with some cases of HD. Most healthy adults are persistently infected with EBV and lymphoid cells are latently infected (Niedobitek *et al.*, 1992, Hummel *et al.*, 1992). Thus, the detection of EBV in any lymphoid tumour raises the possibility that it is merely a passenger virus. Examination of the terminal repeats of the viral genome provides a means to assess the number of infectious events that have taken place within a cell population. This data provides an indirect assay for the clonality of the cells with respect to the virus (Raab-Traub & Flynn, 1986). The ability to demonstrate monoclonal EBV genomes with a tumour population mitigates against the presence of a passenger virus and lends support to the argument that a virus is playing some role in tumour causation.

The relationship between oncogenic DNA viruses and HD is the subject of this thesis.

Chapter 2
General Materials and Methods

This chapter describes the materials and methods that have been routinely used throughout the work described in this thesis. Any additional material and methods required to conduct the experiments described in individual chapters are summarised within the appropriate chapters.

2.1 Materials

The chemical reagents used were purchased from The Sigma Chemical Company Limited (Poole, Dorset, UK) or BDH (Poole, Dorset, UK) except where specifically stated in the text. Deionised water obtained from a reverse osmosis system (Millipore) was used for to make up buffers and solutions. Deionised, filtered water (MilliQ water filtration system, Millipore) was used for all enzymatic reactions and the resuspension of DNA.

2.2 Preparation of samples

2.2.1 Preparation of high molecular weight DNA

Extraction of high molecular weight DNA from tissue samples was carried out in a Containment level II facility within a Class II microbiological safety cabinet. Biopsy samples were disrupted mechanically in TNE buffer (Appendix I), in a Stomacher (Colworth). The cells were pelleted by centrifugation at 2500 revolutions per minute (rpm) (MSE Mistral 1000 centrifuge) for five minutes and further washed in TNE buffer. The cell pellet was resuspended in five millilitres (mL) of TNE buffer. Proteinase K and sodium dodecyl sulphate (SDS) were added to a final concentration of 100μg/mL and 0.5% respectively and the suspension incubated at 55°C for one hour, or at 37°C for 16-20 hours. In order to remove the protein an

equal volume of phenol (Rathburn Chemical Company, Walkerburn, UK) was added and the phases mixed gently by inversion. The aqueous phase was removed following centrifugation at room temperature. An equal volume of chloroform was added and the phases mixed thoroughly. Following centrifugation the aqueous phase was transferred into 99.7% ethanol. High molecular weight DNA was spooled onto a plastic (polypropylene) Pasteur pipette and placed into a sterile microfuge tube (Scotlab Bioscience Limited, Coatbridge, UK). The DNA pellet was washed in 70% ethanol then 95% ethanol and allowed to dry for 2-3 hours. The DNA was resuspended in TE buffer (Appendix I) and incubated at 37°C for 16-20 hours to enable the DNA to dissolve thoroughly. The concentration of DNA was estimated by measuring the OD_{260nm} and OD_{280nm} and determining the quantity of DNA using the equation OD_{260nm} of 1.0 is equal to 50μg/mL of double stranded (ds) DNA. The ratio between the readings at 260nm and 280nm (OD_{260}/OD_{280}) provides an estimate of the purity of the nucleic acid. Pure preparations of DNA and RNA have an OD_{260}/OD_{280} ratio of 1.8 and 2.0, respectively (Sambrook *et al.*, 1989).

2.2.2 Cytospin preparations

Cell lines were maintained in RPMI 1640 medium containing 10% heat-inactivated foetal bovine serum (FBS; heat inactivated at 56°C), 400μg/mL streptomycin, 400U/mL penicillin and 2mM L-glutamine (all obtained from Life Technologies Inc., Paisley, UK). Cells were split at a ratio of 1:5 twice weekly, gassed with 5% CO_2 and incubated in sealed tissue culture flasks (Costar, High Wycombe, UK) at 37°C. Prior to splitting the cells, a one millilitre aliquot was removed, centrifuged and washed three times in Hank's buffered saline solution (HBSS, Life Technologies Inc., Paisley, UK) containing 2% FBS. The number of cells was counted using a

haemocytometer (Fisons, Loughborough, UK) and the cells resuspended at a concentration of 1 x 10^5 cells/mL. A 50µL volume of the cell suspension was centrifuged onto a glass slide (Fisons, Loughborough, UK) at 400rpm (Shandon Cytospin 2) for 5 minutes. The cytospin preparations were air dried and fixed in ice-cold acetone or methanol for 30 minutes. Cell preparations were stored at -20°C.

2.2.3 Paraffin-embedded tissue samples

Three to five micrometre sections from paraffin-embedded biopsy tissues were mounted on 3-aminopropyltriethoxysilane (APES) coated slides (Appendix VI) and stored at room temperature. Prior to analyses, tissue sections were dewaxed in two changes of a xylene substitute, Citroclear (National Diagnostics, Atlanta, Georgia), for a minimum of five minutes. The Citroclear was removed from the tissue sections by washing in two changes of ethanol (99.7%) for five minutes each and then washing in distilled water. Sections which were fixed in a mercuric chloride-based fixative (e.g. formal sublimate) were treated with Lugol's iodine solution (Appendix II) for 1-2 minutes and rinsed in water. The iodine/mercury complex was removed using a 5% solution (w/v) of sodium thiosulphate in deionised water. The sections were rinsed thoroughly in water. Prior to immunohistochemical analyses (section 2.3), the sections were placed in TBS pH 7.6 (Appendix II). In order to examine the localisation of viral nucleic acid using *in situ* hybridisation (section 2.4), following pretreatment with pronase (see sections 2.4.1 and 4.2.3.1), the tissue sections were dehydrated and air dried at room temperature.

2.3 Immunohistochemical methods

2.3.1 Pretreatment of samples

Paraffin-embedded tissue sections were dewaxed and rehydrated as described in section 2.2.3. The sections were washed in two changes of TBS pH 7.6 (Appendix II) for five minutes each. Cell preparations which had been stored at -20°C were allowed to return to room temperature and rehydrated in TBS pH 7.6 for 10 minutes.

2.3.2 Enzymatic digestion

Pretreatment of formalin-fixed, paraffin-embedded tissue with proteolytic enzymes enabled a clearer demonstration of certain antigens. The sections were pre-warmed in TBS pH 7.6 at 37°C for 5-10 minutes. The slides were placed in a 0.1% trypsin/0.1% calcium chloride solution pre-warmed to 37°C over a time period of 20 minutes (section 3.2.2.3). The duration of proteolytic digestion depended on the nature of the fixative and antigen under consideration. The enzymatic reaction was stopped by washing the sections in deionised water and then TBS pH 7.6 for five minutes. Cell preparations fixed in acetone or methanol did not require enzymatic digestion for the detection of antigens.

2.3.3 Microwaving

As an alternative to proteolytic digestion, a microwave oven method for antigen retrieval was utilised. Sections were immersed in 0.01M citrate buffer, pH 6.0 at room temperature. These tissues were heated in a household microwave oven (Swan, compact microwave oven; model 20701) at 750W for 10-20 minutes. After

the heating period, the slides were removed from the hot buffer and placed into deionised water at room temperature.

2.3.4 Primary monoclonal antibody

Care was taken to ensure that the sections did not dry out during the immunohistochemical staining procedure. In order to reduce non-specific staining, filtered normal sheep serum (NSS) at a dilution of 1:5 in TBS pH 7.6 was applied to the tissue sections for 15-30 minutes. Excess NSS was blotted from the tissue sections and the primary monoclonal antibody solution added for a minimum of one hour at room temperature or overnight at 4°C. The optimal working dilution of the monoclonal antibody preparation was assessed for each antibody and each individual tissue tested (section 3.2.2.4). The antibody was diluted in TBS pH 7.6.

2.3.5 Indirect Immunohistochemical staining method

Following incubation of the monoclonal antibody preparation (section 2.3.4), the sections were washed in TBS pH 7.6 for three minutes. The secondary antibody, sheep anti-mouse IgG1 (isotype specific; The Binding Site, University of Birmingham, Birmingham, UK) at a dilution of 1:25 was applied to the tissue sections for 30 minutes. After further washes in TBS pH 7.6 over 10 minutes, a donkey anti-sheep Ig conjugated with alkaline phosphatase (AP) (The Binding Site, University of Birmingham, Birmingham, UK) prepared at a dilution of 1:50 was placed on the tissue sections for 30 minutes. Following further washing in TBS pH 7.6 for 10 minutes, bound alkaline phosphatase (AP) was detected using Fast red TR salt as substrate. This was used at a final concentration of 1mg/mL in AP substrate solution (Appendix II) and filtered directly onto the slides and incubated

for 20 minutes. The enzymatic reaction was stopped by washing in distilled water and the tissue counterstained in aqueous haematoxylin (Mayer's haematoxylin, Appendix II). The haematoxylin stain was differentiated in Scott's Tap Water Substitute (STWS) (Appendix II) and the tissues were mounted using aqueous mountant (Aquamount, BDH). A positive reaction was demonstrated by a red reaction product visualised using light microscopy.

2.3.6 Immunohistochemical staining using an avidin-biotin method

These staining procedures were carried out at room temperature. After incubation of the monoclonal antibody preparation (section 2.3.4), the sections were washed in TBS pH 7.6 for three minutes. The secondary antibody, biotinylated sheep anti-mouse immunoglobulin (isotype-specific) (The Binding Site, University of Birmingham, UK), at a dilution of 1:200, was applied to the tissue sections for 30 minutes. After further washes in TBS pH 7.6 over 10 minutes, the avidin-biotin complex conjugated with alkaline phosphatase (ABC-AP) (Dako Limited, High Wycombe, UK) prepared at a dilution of 1:50 was placed on the tissue sections for 30 minutes. As described in section 2.3.5, bound alkaline phosphatase (AP) was detected using Fast red TR salt as substrate and the tissue sections were counterstained in aqueous haematoxylin (Mayer's haematoxylin, Appendix II).

2.3.7 Immunohistochemical staining using an alkaline phosphatase anti-alkaline phosphatase (APAAP) method

The preparation of tissue samples, enzymatic digestion and primary antibody titrations are described in sections 2.3.1, 2.3.2, and 2.3.4.

Following incubation with the primary monoclonal antibody preparation, the tissues were briefly washed in TBS pH 7.6 for three minutes. Using the methodology described by Cordell *et al.* (1984), the secondary antibody, rabbit anti-mouse immunoglobulin (Dako Limited, High Wycombe, UK) at a dilution of 1:25 was applied to the tissue sections for 30 minutes at room temperature. After washing in TBS pH 7.6, the APAAP complex (Dako Limited, High Wycombe, UK) diluted 1:50 was added to the tissue and incubated for 30 minutes at room temperature. The secondary antibody and APAAP complex steps, with intervening washing in TBS pH 7.6 as described above, were repeated but with a reduction of incubation times to 15 minutes each. Positive reactivity was visualised using Fast red TR salt as described in section 2.3.5. The tissue sections were counterstained in aqueous haemoatoxylin (Mayer's haematoxylin, Appendix II).

2.4 RNA *in situ* hybridisation using oligonucleotide probes

2.4.1 Pretreatment of samples

Three to five micrometre paraffin-embedded tissue sections were processed as described in section 2.2.3. All solutions were made up in diethylpyrocarbonate (DEPC) treated water (Appendix V).

The digestion of paraffin-embedded lymph node tissue sections using pronase (Boehringer Mannheim Limited, Lewes, UK), diluted in the appropriate buffer (Appendix III), was optimised at 1mg/mL for five minutes at room temperature. The proteolytic enzyme reaction was stopped by immersing the sections in glycine buffer (Appendix III). Sections were dehydrated by placing in 70%, 85% and 99.7% ethanol for five minutes each and air dried at room temperature.

2.4.2 3'-end labelling of oligonucleotide probe

Oligonucleotide probes incorporating a biotin molecule at the 5'-end (Alta Bioscience, University of Birmingham) were synthesised on an automated oligonucleotide synthesiser. The concentration of oligonucleotide was calculated by determining the OD_{260nm} and OD_{280nm}. The concentration of the probe was calculated using the formula OD_{260nm} is equal to 30μg/mL for a single stranded (ss) DNA molecule. In a total reaction volume of 50μL, 3.5μg of probe was added to 10μL 5x TdT buffer (0.5M potassium cacodylate pH 7.1, 10mM cobalt chloride, 1mM Dithiothreitol (DTT)), 1.5μL terminal transferase (TdT) enzyme (stock solution 15U/μL, Life Technologies Inc., Paisley, UK) and 16.7μL biotin-11-dUTP (stock solution 0.3mM in Tris pH 7.5, The Sigma Chemical Company, Poole, Dorset, UK). The labelling reaction was carried out at room temperature for four hours. The reaction was stopped by placing on ice. The labelled probe was separated from unincorporated nucleotides using a commercially prepared sephadex-G50 NICK column (Pharmacia Limited, Milton Keynes, UK). The probe was diluted to a final concentration of 8.75ng/μL assuming complete recovery of the oligonucleotide.

2.4.3 Hybridisation of oligonucleotide probe

The optimal hybridisation temperature was calculated as 37°C for the EBV EBER-1 oligonucloeotide probe (see section 4.2.3.2). The tissue sections were incubated with pre-hybridisation buffer (Appendix III) for two hours at 37°C. A final probe concentration of 0.25ng/μL was experimentally-determined to be optimal (section 4.2.3.3) and this was diluted in the hybridisation buffer (Appendix III). The sections were subsequently hybridised for 16-20 hours at 37°C .

2.4.4 Detection of biotinylated probes

All post-hybridisation wash steps were carried out at room temperature. The sections were washed in two changes of 2 x SSC (Appendix I) for 15 minutes each. Two further washes were carried out in 1 x SSC for 15 minutes each.

Bound probe was detected using avidin-biotin complexes conjugated with alkaline phosphatase (ABC-AP). The antibody was prepared in the appropriate buffer (Appendix III) at a dilution of 1:500. This solution was placed on the tissue sections for two hours and incubated at 37°C. The tissue sections were washed three times in wash solution 1 (Appendix III) for three minutes each. An additional wash with solution 2 (Appendix III) was carried out for three minutes. The nitro blue tetrozolium (NBT) at a final concentration of 0.33mg/mL (Appendix III) and bromo-chloro-indolyl phosphate (BCIP) at a final concentration of 0.16mg/mL (Appendix III) in substrate buffer pH 9.0 (Appendix III) were added to the sections for 30-60 minutes. The development of the substrate solution was observed by naked eye and the reaction was stopped by placing the sections in deionised water. The sections were mounted in aqueous mountant (Aquamount, BDH) and in the majority of cases the sections were not counterstained. A positive reaction was identified as a blue-black reaction product visible under light microscopy.

2.5 Growth of bacteria and extraction of plasmid DNA

2.5.1 Transformation of competent cells

Competent bacteria (Life Technologies Inc., Paisley, UK), *Escherichia coli* HB101 or DH5α strains, were thawed on ice and 100μL placed in a Falcon tube (Code no. 2059, Becton Dickinson UK Limited, Cowley, UK). Between ten and one hundred nanograms (ng) of plasmid DNA were added to the cells which were then placed on ice for 30 minutes. Bacteria and plasmid DNA were heat shocked at 42^{o}C for 45 seconds and then placed on ice for 2-3 minutes. After the addition of 900μL of L-broth (Appendix IV) or SOC (Appendix IV), the Falcon tube was placed in a shaking incubator set at 37^{o}C for one hour. One hundred microlitres of the bacteria were plated onto L-agar plates (Appendix IV) containing the appropriate antibiotic (e.g. ampicillin at 50μg/mL) and incubated at 37^{o}C overnight.

2.5.2 Preparation of plasmid DNA

2.5.2.1 Alkaline lysis method

A single bacterial colony was inoculated into 5mL of L-broth (Appendix IV) containing the appropriate antibiotic (e.g. ampicillin 50μg/mL) and incubated overnight at 37^{o}C in the shaking incubator (Fisons, Loughborough, UK). The bacterial culture was then added to 500mL of L-broth (Appendix IV) containing the appropriate antibiotic and further incubated with shaking at 37^{o}C for 16-20 hours.

Bacteria were pelleted using a JS-7.5 rotor (Beckman J2-21 centrifuge, Beckman Instruments Limited, High Wycombe, UK) at 7000rpm for 10 minutes at 4^{o}C. The

supernatant was discarded and the bacterial cells were washed in 100mL STE buffer (Appendix IV). The supernatant was removed after further centrifugation at 7000rpm for 10 minutes. In order to digest the bacterial cell wall, the pellet was resuspended in 4mL of a 0.5% lysozyme solution (solution 1; Appendix IV) and allowed to stand at room temperature for five minutes. The bacterial membrane was lysed and DNA was denatured by adding 16mL of an alkaline solution containing detergent (solution 2; Appendix IV). The mixture was vortexed and incubated on ice for 10 minutes. Plasmid DNA was reannealed by neutralising the solution with 12mL of potassium acetate solution pH 4.8 (solution 3; Appendix IV). The mixture was placed on ice for a further 10 minutes. The lysed bacteria were pelleted by centrifugation at 19000rpm in a JA-20 rotor (Beckman J2-21 centrifuge, Beckman Instruments Limited, High Wycombe, UK) for 30 minutes at room temperature. The supernatant was distributed between two glass Corex tubes. In order to precipitate the plasmid DNA, 0.6 volumes of isopropyl alcohol were added and mixed thoroughly. The resultant solution was centrifuged for 30 minutes at 10000rpm in a JA-20 rotor (Beckman JS-21 centrifuge, Beckman Instruments Limited, High Wycombe, UK) at room temperature. The supernatant was removed and 5mL of 70% ethanol were added to the pellet in order to remove the salt from the precipitated plasmid DNA. This solution was centrifuged at 10000rpm in a JA-20 rotor (Beckman J2-21 centrifuge, Beckman Instruments Limited, High Wycombe, UK) for 15 minutes at room temperature. The pellet was allowed to dry for a maximum of 10 minutes and was then resuspended in 5mL of TE buffer (Appendix I).

The plasmid DNA was purified in a caesium chloride/ethidium bromide gradient. Caesium chloride and ethidium bromide were added at a final concentration of 1mg/mL and 740µg/mL, respectively. The solution was transferred to Beckman

"Quick Seal" centrifuge tubes (Beckman Instruments Limited, High Wycombe,UK). DNA was separated on a density gradient by centrifugation in a Vti 65.2 rotor (Beckman L-8M ultracentrifuge, Beckman Instruments Limited, High Wycombe, UK) at 49000rpm for 16-20 hours at 20°C. DNA bands were visualised using a hand held UV lamp (UVP Inc., San Gabriel, CA, USA, Model UVGL-58) and the supercoiled plasmid identified as the lower band on the gradient. This band was removed using a needle and syringe and was diluted with TE buffer (Appendix I) to a final volume of 5mL. The ethidium bromide was removed with water saturated 1-butanol. When the aqueous phase was colourless, the plasmid DNA was precipitated in 2 volumes of 99.7% ethanol at -20°C for 2 hours. The DNA was pelleted by centrifugation at 10000rpm for 30 minutes at 4°C in a JA-20 rotor (Beckman J2-21 centrifuge, Beckman Instruments Limited, High Wycombe, UK). The pellet was washed in 70% ethanol, allowed to dry and resuspended in TE buffer.

DNA, for use as probe, was digested with the appropriate restriction endonuclease (Life Technologies Inc., Paisley, UK) and electrophoresed on a 0.8% agarose gel in TAE buffer (Appendix I). Ethidium bromide at 0.5μg/mL was added to the gel. The DNA was visualised using a hand held UV lamp (UVP Inc., San Gabriel, CA, USA, Model UVGL-58). Appropriate DNA fragments was purified using DEAE membrane or using a Gelase protocol.

2.5.3 Elution of DNA from DEAE membrane

Using a scalpel blade a small cut was made in the gel directly below the DNA fragment of interest. DEAE membrane (Schleicher & Schuell, West Germany) was placed in the gel space. The gel was run at 50V/cm until the DNA migrated onto the

DEAE membrane. The excess membrane was trimmed and placed in elution buffer (Appendix I) and was incubated at 65°C for one hour. An equal volume of PCI9 (Appendix IV) was added, the mixture was vortexed and then centrifuged at 13000rpm (MSE microcentrifuge, Fisons, Loughborough, UK). DNA was precipitated by addition of 2 volumes of 99.7% ethanol and incubated for two hours at -20°C. The precipitated DNA was collected by centrifugation, washed in 70% ethanol and air dried. The pellet was resuspended in TE buffer (Appendix I). The concentration of DNA was estimated by electrophoresis of an aliquot of the fragment on an agarose gel and comparison with a DNA sample of known concentration.

2.5.3.1 Gelase procedure

The recovery of intact DNA from a 1% low melting point agarose gel (Life Technologies Inc., Paisley, UK) was carried out using a commercial enzyme preparation, Gelase (Cambio Limited, Cambridge, UK). The gel slice containing the DNA fragment of interest was excised from the low melting point agarose gel, weighed and melted at 70°C for 20 minutes with Gelase buffer. The DNA solution was equilibrated by placing at 45°C for 10 minutes. The Gelase enzyme was added to the DNA (stock solution 1U/μL; 1 unit of Gelase enzyme was added for every 100mg of molten 1% low melting point-agarose gel in TAE buffer) and incubated for one hour at 45°C. The eluted DNA was precipitated using 2.5M ammonium acetate (stock solution, 5M ammonium acetate) and centrifuged at 10000rpm in a JA-20 rotor (Beckman J2-21 centrifuge, Beckman Instruments Limited, High Wycombe, UK) for 30 minutes at room temperature. The pellet was resuspended in TE buffer. In order to determine the concentration of DNA, a comparison with DNA of known concentration was performed as outlined above (see section 2.5.3).

2.5.4 Radioactive labelling of dsDNA using random priming

Twenty five nanograms of linear dsDNA were denatured by boiling for five minutes and cooled on ice. Radioactive labelling using the Multiprime Kit (Amersham International plc, Little Chalfont, UK) was carried out according to the manufacturers instructions. In a total reaction volume of 50μL, 10μL of buffer solution containing dATP, dGTP, dTTP, 5μL of primer solution with random hexanucleotides, 3MBq α^{32}P dCTP (800Ci/mmol: Amersham International plc, Little Chalfont, UK) and 2U of the Klenow fragment of DNA polymerase I (stock solution 1U/μL) were incubated for four hours at room temperature. Unincorporated radioactive nucleotides were removed by sephadex column separation. Sephadex-G50 solution (Pharmacia Limited, Milton Keynes, UK) was pipetted into columns and then equilibrated with TE buffer (Appendix I) to enable the sephadex to settle. The multiprime labelling reaction was added and a Geiger counter used to monitor the column. The radioactively labelled DNA probe was detected as the first peak of radioactivity eluted from the column and the unincorporated nucleotides were monitored as the second radioactive peak. The radioactively labelled DNA probe was collected and stored at -20°C.

2.6 Analysis of high molecular weight DNA

2.6.1 Restriction endonuclease digestion of high molecular weight and plasmid DNA

Ten micrograms of high molecular weight DNA were digested with the appropriate restriction endonuclease (Life Technologies Inc., Paisley, UK) in a 50μL volume containing restriction enzyme reaction buffer at the manufacturers recommended concentration and spermidine at a final concentration of 3mM. Five units of restriction enzyme were added per μg of DNA. In a 10μL volume, one microgram of plasmid DNA was digested with a restriction endonuclease. The reactions were incubated at 37°C for 16-20 hours for high molecular weight DNA and for 1-2 hours for digestion of plasmid DNA.

2.6.2 Agarose gel electrophoresis

Restriction endonuclease digested high molecular weight DNA was separated on a 0.8% agarose gel in TBE buffer (Appendix I). The DNA samples and one tenth volume of gel running dye (Appendix I) were loaded into the wells. The high molecular weight DNA marker, Lambda DNA digested with Hind III (Life Technologies Inc., Paisley, UK), was loaded adjacent to the sample DNA to enable sizing of the fragments. The samples were electrophoresed at 32V/cm for 16-20 hours and the gel was immersed in ethidium bromide at a final concentration of 0.5μg/mL. The DNA was visualised on a transilluminator (Spectroline transilluminator, Model TC-254A) and photographed with a Polaroid MP4 Land Camera with Polaroid Type 57 high speed film (Genetic Research Instrumentation Limited, Dunmow, UK).

2.6.3 Southern blotting procedure

High molecular weight DNA was transferred to nylon membrane (Hybond-N, Amersham International plc, Little Chalfont, UK) using a modified Southern blotting procedure. After photography, the DNA was denatured by placing in alkali buffer (Appendix I) for one hour at room temperature. This solution was replaced by neutralising buffer (Appendix I) and incubated for a further one hour. The gels were placed in transfer buffer, 10 x SSC (Appendix I). The DNA was blotted downward using capillary action assisted by gravity. The nylon membrane and gel were placed on a layer of absorbent material and overlaid with a sponge soaked in transfer buffer. The blots were sealed in plastic bags to avoid evaporation and left for 16-20 hours. The nylon membrane was rinsed in 3x SSC to remove any extraneous material and the DNA was irreversibly bound to the membrane by cross-linking with UV light (UV Stratalinker 1800, Stratagene Limited, Cambridge, UK).

2.6.4 Hybridisation of Southern blots

The nylon filters were soaked in 3x SSC, 0.1% SDS and placed, with the bound DNA facing inside, in a glass tube suitable for the hybridisation oven (Techne Hybridiser HB-1, Philip Harris Scientific, Clydebank, UK). The filters were prehybridised with 10mL of the hybridisation buffer for 4 hours at 65°C with continuous rotation. The labelled probe (see section 2.5.4) was boiled for five minutes and cooled rapidly on ice. The ^{32}P-labelled probe was added to the hybridisation buffer and the filters were rotated at 65°C for 16-20 hours. The filters were washed initially in two changes of 2x SSC, 0.1% SDS at room temperature for five minutes each. The filters were washed in two changes of 0.5x SSC, 0.1% SDS at 65°C for 30 minutes each. The filters were wrapped in Saran wrap (Genetic

Research Instrumentation Limited, Dunmow, UK) and autoradiographed in a cassette containing an intensifying screen. The autoradiographs at -70°C were developed after exposures of 1-21 days as required.

Chapter 3

Expression of the EBV encoded latent gene product LMP-1 in Hodgkin's disease; lack of correlation with CD23 and bcl-2 expression

3.1 Introduction

The human herpesvirus, EBV has been linked with a number of malignant conditions including BL (Henle *et al.*, 1969, zur Hausen *et al.*, 1970, Nonoyama *et al.*, 1973, 1974), lymphomas in immune deficient persons (Purtilo *et al.*, 1985, Loning *et al.*, 1987), NPC (zur Hausen *et al.*, 1970, Nonoyama *et al.*, 1973) and more recently HD.

Using molecular techniques EBV genomes have been detected in HD biopsies from 17-41% of cases (Weiss *et al.*, 1987a, Anagnostopoulos *et al.*, 1989, Staal *et al.*, 1989, Boiocchi *et al.*, 1989, Jarrett *et al.*, 1991a). Although EBV was detected in lesions in a proportion of HD cases, it was unclear what role, if any, the virus was playing in the disease process. In order to address this issue the clonality of the EBV-infected cells was examined. Analysis of the terminal repeat region of the EBV genomes indicated that in the majority of cases examined the EBV was monoclonal (Weiss *et al.*, 1987a, Anagnostopoulos *et al.*, 1989, Gledhill *et al.*, 1991, Jarrett *et al.*, 1991a). These above results support the suggestion that in a proportion of HD cases the EBV had infected a single cell, which had clonally expanded.

At the initiation of this work the localisation of EBV within the affected tissues of HD had not been fully determined. Furthermore, there were no data regarding the expression of EBV latent gene products in HD biopsies with the exception of one early case report. Poppema *et al.* (1985) used human sera to demonstrate expression of an EBV nuclear antigen in HD. Recently monoclonal antibodies became available for EBV latent gene products LMP-1 and EBNA-2 (Rowe *et al.*, 1987, Young *et al.*, 1989).

LMP-1 is the only virus encoded protein which has been shown to have transforming ability, in isolation. In B-cell lines, LMP-1 is known to upregulate the cellular adhesion molecules, LFA-1, LFA-3 and ICAM-1 and the activation antigen CD23 (Wang *et al.*, 1988, 1990).

Recent data suggest that the expression of LMP-1 can protect B-cells from programmed cell death, apoptosis (Gregory *et al.*, 1991). This effect is mediated by the LMP-1 induced upregulation of the bcl-2 gene (Williams *et al.*, 1990, Henderson *et al.*, 1991). The t(14;18) translocation, which is present in more than 85% of follicular lymphomas, is also thought to cause an increased expression of the bcl-2 gene (Weiss *et al.*, 1987b, Cotter, 1990). One report has indicated that this translocation occurs in 32% of HD cases (Stetler-Stevenson *et al.*, 1990). It was therefore speculated that bcl-2 expression, induced by either LMP-1 or by the translocation t(14;18), might play a role in HD.

This study was designed to further investigate the role of EBV in HD through the examination of EBV latent genes in RS cells. Using immunohistochemical methods, the localisation of the EBV latent gene product LMP-1 was examined in a series of HD cases previously analysed for the presence of EBV genomes. In a limited number of cases the expression of EBNA-2 was also studied. The expression of EBV LMP-1 was subsequently correlated with the expression of the activation antigen, CD23 and bcl-2 protein.

3.2 Materials and Methods

3.2.1 Clinical samples

Lymph node biopsies from HD patients were referred to the Leukaemia Research Fund (LRF) Virus Centre from a number of clinical centres for virological analyses. Thirty four HD cases were included in this study. Paraffin-embedded lymph node biopsy material was available from all of the cases. Frozen material, stored at the Department of Pathology, University of Edinburgh Medical School, was available from eleven cases. DNA from these samples had been examined previously for the presence of EBV genomes using Southern blot analysis. In the EBV positive cases the clonality of the EBV-infected cells was assessed (Gledhill *et al.*, 1991, Jarrett *et al.*, 1991a). Selection for this study was based on the EBV status of cases; seventeen cases contained clonal EBV genomes and seventeen cases were EBV negative. Following histological review, the cases were classified according to the Rye classification (Lukes *et al.*, 1966). The series comprised 12 nodular sclerosis HD (NSHD), 18 mixed cellularity HD (MCHD), 2 lymphocyte depleted HD (LDHD) and 2 lymphocyte predominant HD (LPHD) cases. Three reactive nodes were included as negative controls.

3.2.2 Immunohistochemical analysis

3.2.2.1 Monoclonal antibodies

The cocktail of monoclonal antibodies, CS1-4, specific for EBV LMP-1 was kindly provided by Professor L.S. Young, University of Birmingham, Birmingham, UK. The individual monoclonal antibodies, CS1, CS2 and the combined CS3 and CS4 were shown to recognise three different epitopes on the EBV LMP-1 protein (Rowe *et al.*, 1987). A monoclonal antibody specific for LMP-1, S12, which recognises an epitope distinct from those recognised by the CS1-4 monoclonal antibodies was provided by Professor D. Thorley-Lawson (Mann *et al.*, 1985). The PE2 monoclonal antibody specific for the EBNA-2 protein was also donated by Professor L.S. Young (Young *et al.*, 1989). The monoclonal antibody bcl-2 100 was kindly provided by Dr D.Y. Mason, LRF Immunodiagnostics Unit, John Radcliffe Hospital, Oxford, UK (Pezzella *et al.*, 1990). The monoclonal antibody BU38 specific for the CD23 molecule was obtained from The Binding Site, University of Birmingham, Birmingham, UK.

3.2.2.2 Detection of EBV LMP-1

Using the monoclonal antibodies, CS1-4, a series of experiments was carried out to ascertain whether these antibodies could be used on paraffin-embedded lymph node material. Optimisation experiments were carried out on cytospin preparations of L591, an EBV-positive HD derived cell line and J-Jhan, a T-cell line (prepared as in section 2.2.2) and subsequently on paraffin-embedded sections from a case of NSHD shown to be EBV-positive using the CS1-4 antibodies in early experiments.

3.2.2.3 Enzymatic digestion

As described in section 2.3.2, the trypsin digestion of paraffin-embedded lymph node sections was tested over time point varying from 0-20 minutes. The intensity of staining within RS cells using the CS1-4 antibody in paraffin-embedded tissue was considerably greater without trypsin digestion. Recent experiments using heat treatment of paraffin-embedded lymph node tissue in a microwave oven for 10 minutes (see section 2.3.3), prior to addition of the CS1-4 antibodies, resulted in a clearer demonstration of LMP-1 expression in RS cells.

3.2.2.4 Primary antibody

Non-specific staining was observed in initial experiments therefore the monoclonal antibody CS1-4 was purified by pre-adsorbing with HL60 cells which was kindly performed by Dr G. Brown at the Department of Immunology, University of Birmingham). During the following analyses, both the unpurified and purified monoclonal antibody preparations were compared. The cell preparations/sections were incubated for one hour with a series of dilutions of the monoclonal antibody cocktail (CS1-4) from 1:10-1:100. Optimal staining conditions were observed when the tissue sections were incubated with the purified CS1-4 monoclonal antibodies at a dilution of 1:50 for 60 minutes at room temperature.

3.2.2.5 Detection of antibody using immunohistochemical methods

Due to problems with background staining four different methods of detecting the antigen/antibody complex were investigated (Figure 3.1).

1. an indirect staining procedure (section 2.3.5) using sheep anti-mouse IgG1 (isotype specific) as secondary antibody and detection with donkey anti-sheep Ig conjugated with alkaline phosphatase (AP) (The Binding Site, Birmingham, UK).

2. an ABC method using a biotinylated anti-mouse IgG1 (The Binding Site, Birmingham, UK, see section 2.3.6) as secondary antibody and detected with an avidin-biotin-AP complex (Dako Limited, High Wycombe, UK).

3. a standard APAAP method (Dako Limited, High Wycombe, UK, as described in section 2.3.7 and Cordell *et al.*, 1984) using rabbit anti-mouse immunoglobulin (Ig) as secondary antibody.

4. a modified APAAP method using an isotype-specific secondary antibody (anti-mouse IgG1, The Binding Site, Birmingham, UK).

The standard APAAP method as described above (section 2.3.7) and the avidin-biotin alkaline phosphatase method (ABC-AP, see section 2.3.6) incorporating the isotype-specific secondary antibody (The Binding Site, Birmingham, UK) were equally sensitive methods of detecting reactivity with the monoclonal antibody cocktail, CS1-4. The ABC-AP method was the most specific method of detecting LMP-1 in paraffin-embedded tissue sections. The IgG1-specific

Figure 3.1 Immunohistochemical staining methods

Immunohistochemical staining methods. Indirect immunohistochemical method, enzyme-labelled tertiary antibody specific to the secondary antibody. Avidin-biotin staining method, preformed avidin-biotin complex labelled with alkaline phosphatase (AP) react with biotinylated secondary antibody. APAAP method, preformed complex reacts with secondary antibody. Primary antibody and antibody of the enzyme immune complex must be made in the same species.

Key for figure 3.1:

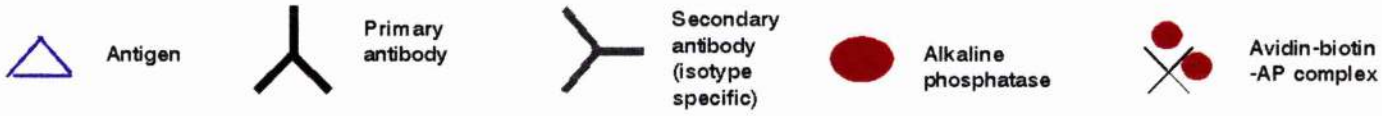
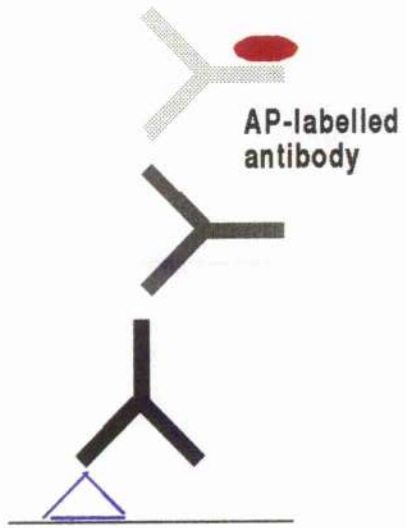
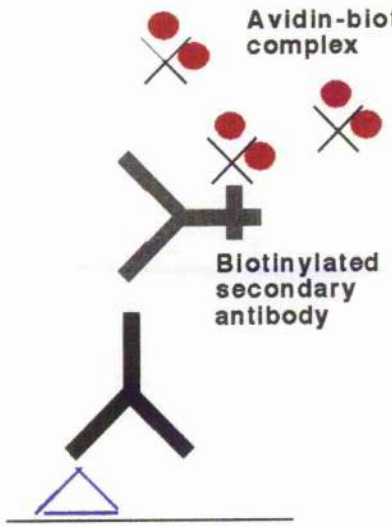


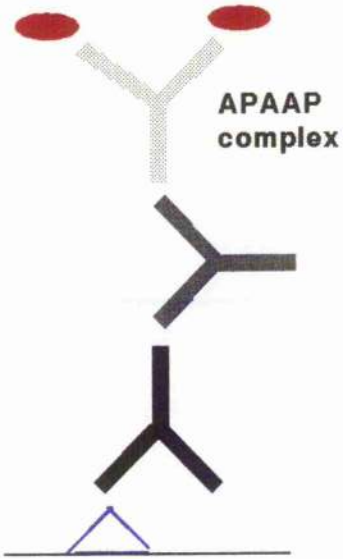
Figure 3.1 Immunohistochemical staining methods



Indirect immunohistochemical staining method



Avidin-biotin staining method



APAAP staining method

secondary antibody increased the specificity of the staining. In subsequent analyses reactivity was thus detected using this methodology.

Selected cases (cases 74 and 75) were also stained with the S12 monoclonal antibody (isotype IgG2a) which recognises another epitope on the LMP-1 molecule, using an APAAP technique as described in section 2.3.7.

3.2.2.6 Controls

The LMP-1 positive NSHD case, identified in initial experiments, was included in each assay as a positive control. One section from each case was stained in parallel with a monoclonal antibody to rotavirus (RC3.6, kindly provided by Dr G. Brown, University of Birmingham) in order to control for non-specific staining. Where excess non-specific staining was encountered the procedure was repeated with 0.1% bovine serum albumin added to the wash buffer, TBS pH 7.6

3.2.3 Detection of EBNA-2

The expression of EBNA-2 was examined on frozen material from two cases (cases 390 and 387) of HD using the PE2 monoclonal antibody and an APAAP method (section 2.3.7). Paraffin-embedded tissue sections from a further three cases of HD (cases 387, 938 and 954) were examined for EBNA-2 expression following reports of enhanced staining with the PE2 antibody after microwave treatment. The tissue sections were incubated in 0.01M citrate buffer pH 6 in a microwave oven for 10 minutes (see section 2.3.3). Cytospin preparations and paraffin-embedded cell pellets of Raji, an EBV-infected cell line and J-Jhan cells were included as positive and negative controls, respectively.

3.2.4 Detection of CD23

Sections from paraffin-embedded material were investigated for the expression of CD23 using the monoclonal antibody BU38 and an APAAP method as described in section 2.3.7.

As described in section 3.2.2.3, prior to incubation with the monoclonal antibody BU38, paraffin-embedded sections were trypsin digested. Optimal staining was obtained following trypsin digestion for 20 minutes at 37°C. Incubation with the primary antibody, BU38 at a dilution of 1:50, was carried out overnight at 4°C. A section from each case was assayed using the antibody to rotavirus as a negative control.

A subset of eleven cases from which frozen material was available were analysed for the expression of CD23 using the monoclonal antibody MHM6 and an immunoperoxidase method. This assay was carried out at the Department of Pathology, University of Edinburgh Medical School .

3.2.5 Detection of bcl-2

Prior to microwave treatment of paraffin-embedded material, staining of paraffin-embedded tissue using the bcl-2 monoclonal antibody, bcl-2 100, was unreliable. The expression of bcl-2 was assayed in eleven cases from which frozen material was available. Frozen sections were stained, at the Department of Pathology, University of Edinburgh Medical school, using an indirect immunoperoxidase technique and a monoclonal antibody to bcl-2 protein (bcl-2 100) (Salter *et al.*, 1985).

3.3 Results

3.3.1 Expression of EBV LMP-1

The correlation between EBV status, assessed by Southern blot analysis, and LMP-1 expression in 34 HD cases is summarised in Tables 3.1 and 3.2. EBV LMP-1 was present in the RS cells and in mononuclear variants in 15 cases (Figures 3.2a and 3.3). In many cases the staining was focal. The pattern of staining was predominantly cytoplasmic although some cells showed a staining pattern consistent with the membrane distribution of the LMP-1 protein. Considerable variation was observed in the proportion of abnormal cells which stained positively within an individual biopsy. In some cases only a minority of the RS cells showed reactivity with the LMP-1 antibodies. Staining was intense even when LMP-1 expression was limited to a few cells in the biopsy. EBV LMP-1 was not detected in the small lymphocytes or other cell types in the reactive infiltrate. No reactivity was noted in sections from the three reactive nodes or in control sections labelled with the antibody to rotavirus (Figure 3b).

3.3.1.1 Southern blot positive HD cases

Seventeen cases containing clonal EBV genomes were analysed for the presence of the EBV LMP-1 protein. This EBV encoded latent gene product was detected in twelve of the EBV-positive HD cases (Table 3.1).

Table 3.1 Expression of EBV LMP-1, EBNA-2, CD23 and bcl-2 in Hodgkin's disease

Southern blot positive HD cases

LRF case number	Age (years)	HD subtype	Southern blot result	LMP-1	EBNA-2	CD23	bcl-2
70	36	LP	C	++	NT	-	NT
79	48	MC	C	++	NT	-	NT
386	80	MC	C	++	NT	-	+
387	14	MC	C	++	-	-	+
390	68	NS	C	++	NT	-	-
937	14	MC	C	++	NT	+	-
938	6	NS	C	++	-	-	NT
940	5	MC	C	++	NT	-	NT
954	31	MC	C	++	-	-	NT
960	59	NS	C	-	NT	-	NT
962	54	NS	C	-	NT	-	NT
974	82	NS	C	++	NT	-	-
981	85	MC	C	++	NT	+	+
982	78	MC	C	-	NT	-	-
987	65	LP	C	-	NT	-	NT
990	67	LD	C	++	NT	-	+/-
998	7	MC	C	++	NT	-	NT

C, clonal EBV genomes; NT, not tested; ++, strongly positive; +, positive; -, negative

NS, nodular sclerosis HD; MC, mixed cellularity HD; LD. lymphocyte depleted HD; LP, lymphocyte predominance HD

Table 3.2 Expression of EBV LMP-1, CD23 and bcl-2 in Hodgkin's disease

Southern blot negative HD cases

LRF case number	Age (years)	HD subtype	LMP-1	CD23	bcl-2
71	22	NS	-	-	NT
74	20	MC	++	-	NT
75	25	NS	++	-	NT
420	19	LD	-	-	NT
430	67	NS	-	-	NT
237	25	MC	-	-	NT
939	7	NS	-	-	NT
951	21	MC	-	-	-
955	24	MC	-	-	NT
956	25	MC	-	-	NT
959	30	MC	-	-	NT
963	54	NS	-	-	NT
964	56	NS	-	-	NT
979	61	MC	-	-	NT
983	60	MC	-	-	++
984	82	MC	-	-	++
995	39	NS	-	-	NT

NT, not tested, ++ strongly positive, + positive, - negative

NS, nodular sclerosis HD; MC, mixed cellularity HD; LD, lymphocyte depleted HD; LP, lymphocyte predominance HD

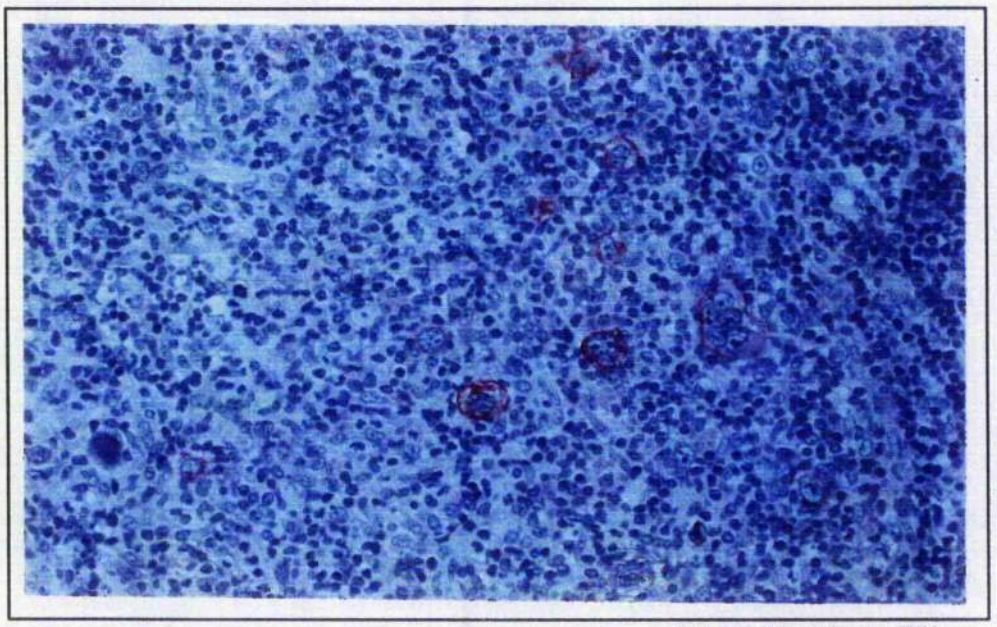
Figure 3.2a: EBV-LMP-1 expression in HD:Case 390

Paraffin-embedded lymph node biopsy section from a 68 year old MCHD case. Clonal EBV genomes. Reactivity with LMP-1 monoclonal antibody (CS1-4) is detected. Clonal EBV genomes were detected. Alkaline phosphatase and Fast red salt, counterstained with haematoxylin.

Figure 3.2b: Immunohistochemical staining with rotavirus monoclonal antibody (RC3.6):Case 390

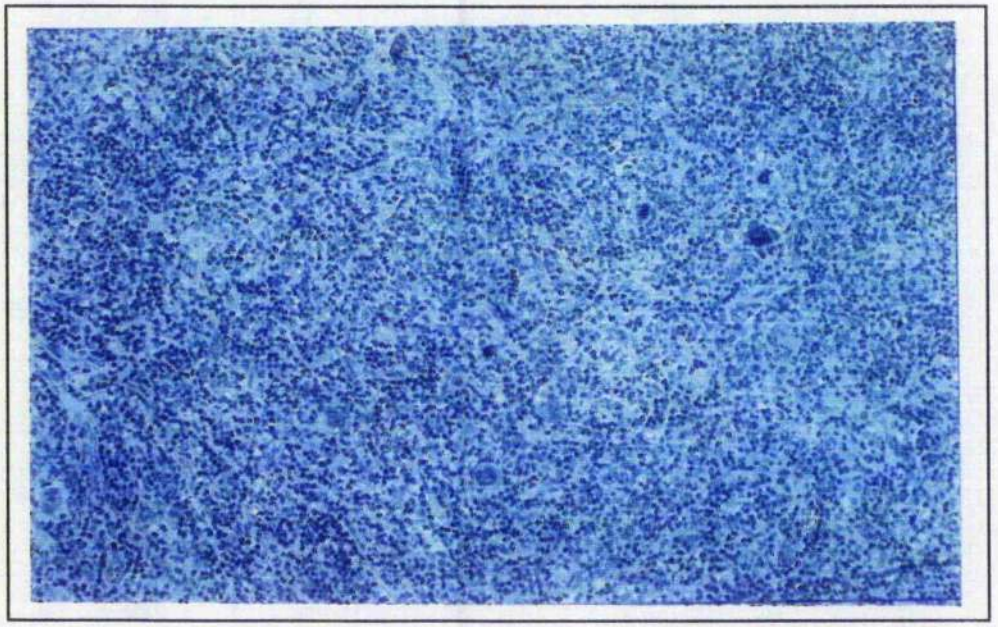
Paraffin-embedded lymph node section from a 68 year old MCHD case. Immunohistochemical staining using rotavirus monoclonal antibody (RC3.6) as negative control. Alkaline phosphatase and Fast red salt, counterstained with haematoxylin.

Figure 3.2a Expression of EBV LMP-1 in HD
Case 390



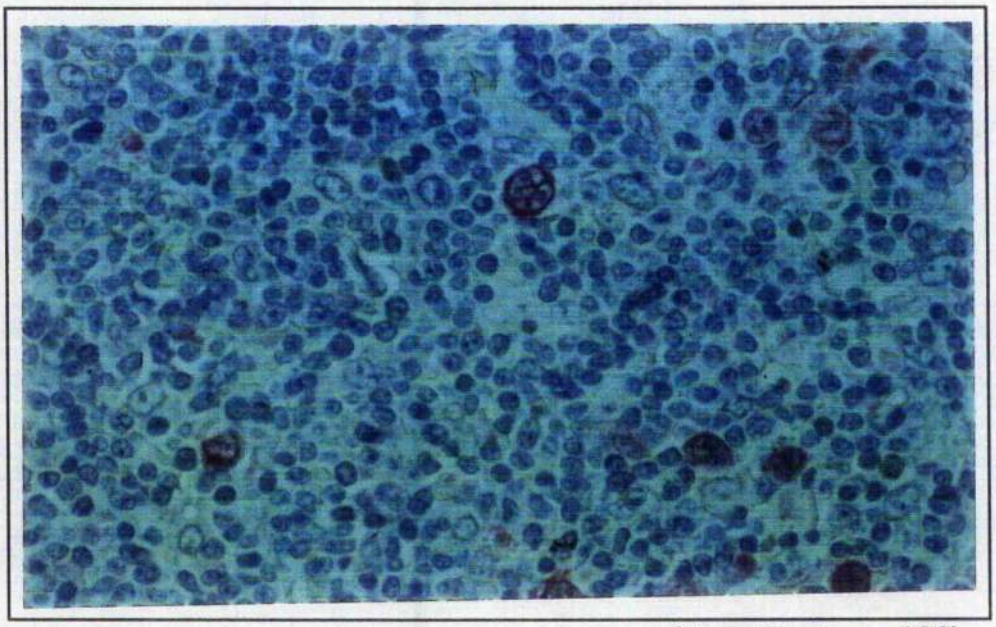
(Magnification x50)

Figure 3.2b Immunohistochemical staining with rotavirus monoclonal antibody (RC3.6)
Case 390



(Magnification x25)

Figure 3.3 Expression of EBV LMP-1 in HD Case 954



(Magnification x100)

Paraffin-embedded lymph node section from a 31 year old MCHD case. Reactivity with the LMP-1 monoclonal antibody (CS1-4) is detected in RS cells. Clonal EBV genomes were detected. Alkaline phosphatase and Fast red salt, counterstained with haematoxylin.

3.3.1.2 Southern blot negative HD cases

The expression of LMP-1 was investigated in seventeen EBV negative HD cases. Two of these, cases 74 and 75, had detectable EBV LMP-1 in the RS cells of the biopsy (Figures 3.4 and 3.5, Table 3.2). Staining of cells in these cases was also observed using the S12 monoclonal antibody confirming the specificity of the staining.

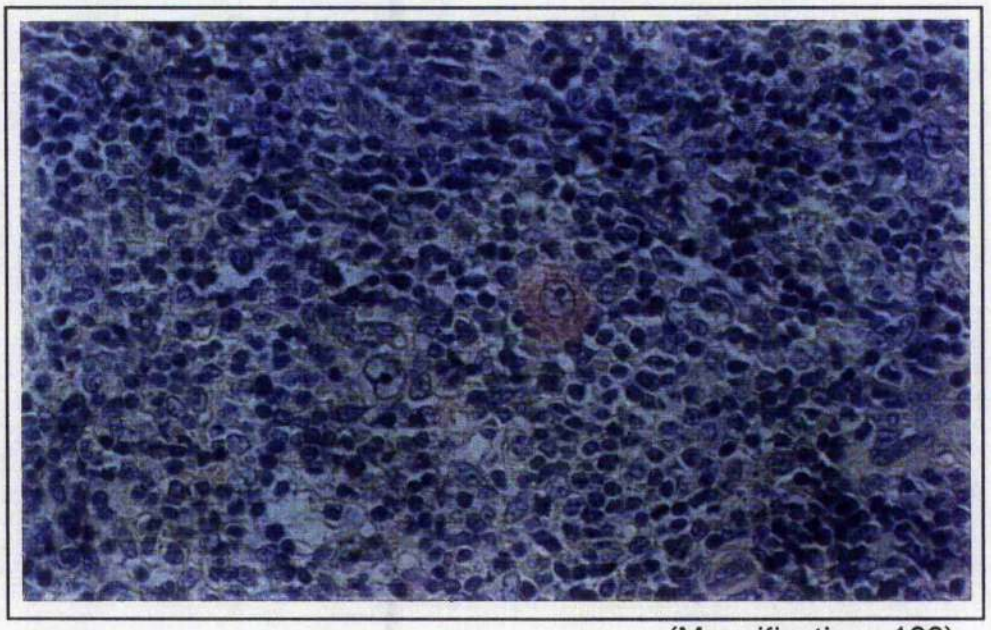
3.3.2 Expression of EBNA-2

Sections from five HD cases were investigated for the presence of EBNA-2 expression. Staining was not detected in any of the five HD cases using the PE2 monoclonal antibody (Tables 3.1 and 3.3). A distinct nuclear pattern of reactivity was observed in EBV-positive cells in positive control material.

3.3.3 Expression of CD23

Sections from all cases were analysed for the presence of CD23 but staining of RS cells was not observed using paraffin-embedded biopsy material (Tables 3.1 and 3.2). In many cases strong staining was observed within the germinal centres of residual follicles (Figure 3.6) thus providing an internal positive control. Using frozen biopsy material, CD23 was detectable in the RS cells in two of the eleven cases examined (Table 3.3).

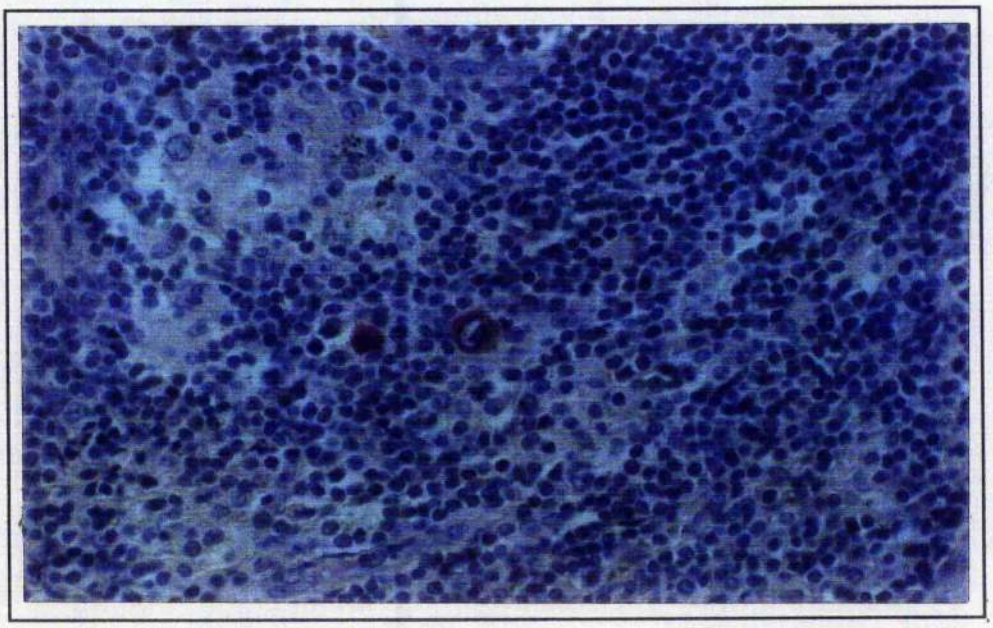
Figure 3.4 Expression of EBV LMP-1 in HD
Case 75



(Magnification x100)

Paraffin-embedded lymph node section from a 24 year old NSHD case. Reactivity with LMP-1 monoclonal antibody (CS1-4) is detected in RS cells. EBV-negative by Southern blot hybridisation. Alkaline phosphatase and Fast red salt, counterstained with haematoxylin.

Figure 3.5 Expression of EBV LMP-1 in HD Case 74



(Magnification x100)

Paraffin-embedded lymph node section from a 20 year old MCHD case. Reactivity with the LMP-1 monoclonal antibody (CS1-4) is detected in RS cells. EBV-negative by Southern blot hybridisation. Alkaline phosphatase and Fast red salt, counterstained with haematoxylin.

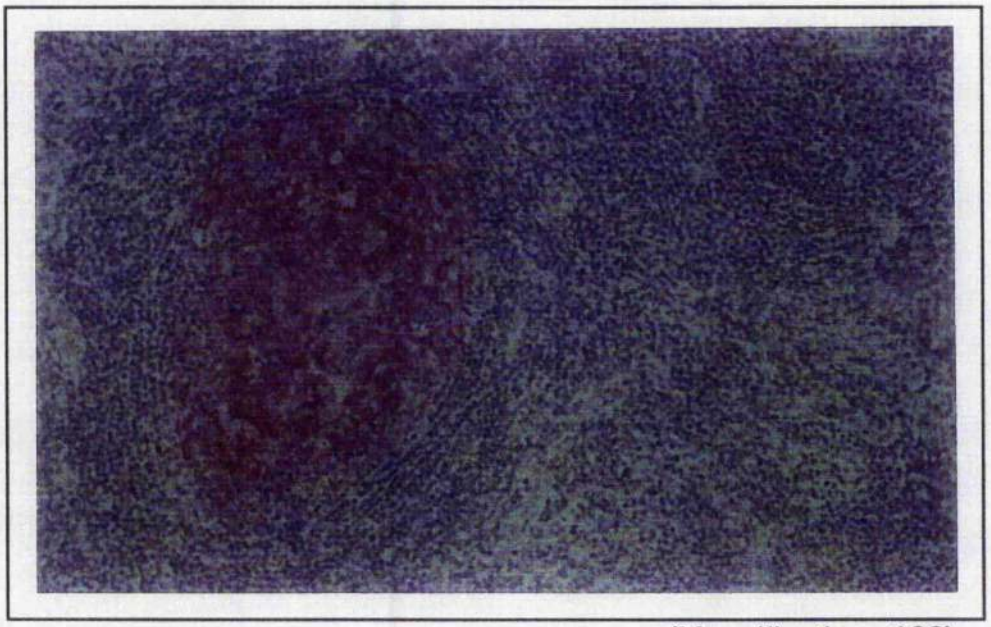
Table 3.3 Detection of EBV LMP-1, EBNA-2, CD23 and bcl-2 in RS cells in frozen biopsy material available from 11 cases of Hodgkin's disease

LRF case number	Age (years)	HD subtype	Southern blot result	LMP-1	EBNA-2	CD23	bcl-2
386	80	MC	C	++	NT	-	+
387	14	MC	C	++	-	-	+
390	68	NS	C	++	-	-	-
937	14	MC	C	++	NT	+	-
951	21	MC	-	-	NT	-	-
974	82	NS	C	++	NT	-	-
981	85	MC	C	++	NT	+	+
982	78	MC	C	-	NT	-	-
983	60	MC	-	-	NT	-	+
984	82	MC	-	-	NT	-	+
990	67	LD	C	++	NT	-	+/-

C, clonal EBV genomes; ++, strongly positive; +, positive; +/-, equivocal; -, negative; NT, not tested

NS, nodular sclerosis HD; MC, mixed cellularity HD; LD, lymphocyte depleted HD

Figure 3.6 Immunostaining of paraffin-embedded HD biopsy material using the BU38 monoclonal antibody specific for CD23



(Magnification x100)

Staining of paraffin-embedded HD biopsy material using the BU38 monoclonal antibody. Intense staining within the germinal centres of residual follicles is detected, however no staining is observed in the RS cells. Alkaline phosphatase and Fast red salt, counterstained with haematoxylin.

3.3.4 Expression of LMP-1 and CD23

The correlation between LMP-1 and CD23 expression was analysed for the subset of 11 HD cases from which there was frozen biopsy material available (Table 3.4a). CD23 expression was observed in two of seven cases in which EBV LMP-1 had been detected. In comparison there was no detection of CD23 expression in the RS cells in the EBV negative cases.

3.3.5 Expression of bcl-2

Sections from eleven HD cases were analysed for the expression of bcl-2 (Tables 3.1, 3.2 and 3.3). Cytoplasmic staining of RS cells was seen in five cases. In these cases variable numbers of bcl-2 positive and negative RS cells were present. Small lymphocytes stained with bcl-2 in all the cases examined and, where present, there was no staining in the germinal centres.

3.3.6 Expression of LMP-1 and bcl-2

bcl-2 expression was found in four of the eight cases in which clonal EBV genomes had been detected previously. However, bcl-2 expression was detected in two of the three cases which were EBV negative by Southern blot analysis. The relationship between LMP-1 and bcl-2 expression was analysed and is shown in Table 3.4b. From seven LMP-1 positive cases, three cases expressed the bcl-2 protein within the RS cells.

Table 3.4a Correlation between the expression of EBV LMP-1 and CD23 in RS cells

CD23 status	HD cases	LMP-1 positive	LMP-1 negative
positive	8	2	0
negative	3	5	4
Total cases	11	7	4

Table 3.4b Correlation between the expression of EBV LMP-1 and bcl-2 in RS cells

bcl-2 status	HD cases	LMP-1 positive	LMP-1 negative
positive	8	3	2
negative	3	4	2
Total cases	11	7	4

3.4 Conclusions

The main finding from this study is that immunohistochemical staining using antibodies against the LMP-1 protein clearly shows that EBV is localised to the RS cells in HD. Furthermore, an EBV encoded latent gene product with known oncogenic potential (see section 1.4.3.3), is expressed by RS cells. Previous reports of EBV genomes in HD have now been confirmed by the presence of LMP-1 exclusively in the RS cell population. These results are the first indication that the presence of EBV in HD is likely to be an important step in the pathogenesis of the disease.

During the course of this work two similar studies were published in which EBV LMP-1 was detected in RS cells in frozen biopsy material from a proportion of HD cases. Using the cocktail of monoclonal antibodies CS1-4, Pallesen *et al.* (1991a) investigated 84 HD cases and demonstrated LMP-1 in RS cells in 40 cases (48%). In a similar study, LMP-1 expression was detected in 18 of 47 HD cases (38%) using a polyclonal rabbit antisera directed against LMP-1 (Herbst *et al.*, 1991). Although the biochemical activity of LMP-1 is unknown the presence of a translated latent gene within the malignant cells of a proportion of HD cases has led us to speculate that EBV is aetiologically involved in these HD cases.

Using the PE2 antibody on frozen biopsy tissue from a small number of HD cases, EBNA-2 expression was not detected in RS cells. These results have been confirmed by other groups (Pallesen *et al.*, 1991a, Herbst *et al.*, 1991a). Cattoretti *et al.*, (1993) recently reported a microwave method of antigen unmasking which enabled the use of a number of antibody preparations on formalin-fixed, paraffin-embedded tissue sections. Following microwave

treatment, enhanced staining using the PE2 antibody has been documented (G. Niedobitek, personal communication). Further study of HD cases using this method of antigen retrieval and the PE2 antibody confirmed the above findings.

Selection of cases for this study on the basis of Southern blot results has enabled the correlation of the immunohistochemical analysis with EBV status (Jarrett *et al.*, 1991a, Gledhill *et al.*, 1991). In the majority of cases in which clonal EBV genomes were detected LMP-1 was expressed in RS cells. However, there were four cases in this category in which the LMP-1 protein was not detected. Explanations for the failure to detect expression include that LMP-1 may not have been expressed or was expressed at low levels in the malignant cells and the differences in fixation may have resulted in the negative LMP-1 status of these cases. Cases were referred from various pathological centres thus fixation was not standard.

In addition we identified two cases (cases 74 and 75) which were negative by Southern blot analysis but expressed LMP-1 in RS cells. It would seem unlikely that the detection of EBV in these cases would have been below the detection limit of the Southern blot technique as it is possible to detect a single EBV genome in <0.45% of the cells using this method. These cases were reviewed independently by Dr A.S. Jack, Consultant Pathologist, University of Leeds. In case 74 a large reactive component was described in the biopsy. This would suggest that the numbers of RS cells in this sample may have been insufficient to allow detection of viral genomes by the Southern blot method. In case 75 large numbers of abnormal cells were noted in sections from this biopsy. The most likely explanation for the discrepancy between the Southern blot and immunohistochemical results was that sampling error may have occurred.

Overall, there was a good correlation between the detection of EBV using immunohistochemical analysis and Southern blot hybridisation. The correlation between the detection of EBV using Southern blot analysis and immunohistochemical techniques is discussed further in Chapter 4.

Using sensitive immunohistochemical methods, we have demonstrated that the antibody cocktail CS1-4 could be reliably used in archival paraffin-embedded biopsy material to detect LMP-1 within RS cells, particularly when the numbers of RS cells are low. Pallesen *et al.* (1991a) examined a small number of paraffin-embedded sections and reported weak reactivity with the LMP-1 antibodies in RS cells. In the present study the percentage of LMP-1 positive HD cases detected in paraffin-embedded tissue was comparable to that seen in studies using frozen biopsy material (Pallesen *et al.*, 1991a, Herbst *et al.*, 1991a). Murray *et al.* (1992a) confirmed our findings with the detection of LMP-1 protein in RS cells in 22 out of 46 HD cases (48%) in paraffin-embedded biopsy material.

LMP-1 has also been shown to be a potential target for cytotoxic T cells (Murray *et al.*, 1988). In a normal functioning immune system one would expect these cells would have been removed by cytotoxic T-cells. Therefore, the presence of cells expressing LMP-1 in HD suggests either an impairment or lack of antigen presentation or a defect in cell-mediated immunity.

The identification of LMP-1 protein in RS cells led us to investigate the expression of CD23 and bcl-2, both of which have been shown to be upregulated by LMP-1. LMP-1 is known to induce the expression of CD23 in B cells *in vitro*. CD23 expression was not detected in RS cells in paraffin-embedded sections,

but was detected in the malignant cells in a small number of cases using frozen biopsy material. From these results it is clear that the latter assay is more sensitive. However, these results do suggest that CD23 is present in RS cells in only a minority of cases and is not expressed at high levels (Jarrett *et al.*, 1991b). These findings are consistent with those of other studies (Pallesen, 1987, Herbst *et al.*, 1991a, Sandvej *et al.*, 1993). In contrast, a prior study by Rowlands *et al.* (1990) indicated that CD23 was expressed in RS cells in 13 out of 15 HD cases in paraffin-embedded tissue. From our data and those of other groups we suspect that there is an over-estimation of CD23 expression within the malignant cells in these particular cases.

Further analysis of the correlation between LMP-1 and CD23 expression was limited due to the small numbers of cases investigated using the frozen section assay. Both of the CD23 positive cases were LMP-1 positive therefore we cannot rule out the possibility that in a small proportion of cases the expression of LMP-1 upregulates CD23 in RS cells. Transfection of the EBV-negative BJAB cell line with both EBNA-2 and LMP-1 resulted in increased levels of CD23 compared with cells expressing EBNA-2 and LMP-1 individually (Wang *et al.*, 1990). Failure to up-regulate CD23 in RS cells may therefore be due to the lack of EBNA-2 expression.

Previous studies have examined the presence of the t(14;18) translocation in HD cases using the polymerase chain reaction (PCR). The bcl-2-associated translocation has been detected in 5-32% of HD cases (Stetler-Stevenson *et al.*, 1990, Masih *et al.*, 1991, Reid *et al.*, 1993), however other laboratories have failed to detect the presence of the translocation in HD material (Said *et al.*, 1991, Louie *et al.*, 1991). In our laboratory, the t(14;18) translocation was

identified in only three of fifty HD cases studied (Alice Gallagher, personal communication). The expression of bcl-2 was examined in most of the cases included in the molecular analyses. In the present study, the bcl-2 protein was detected in RS cells in approximately half of the cases examined. The presence of bcl-2 in RS cells did not correlate with LMP-1 positivity. bcl-2 expression in RS cells has been described previously in a variable proportion of HD cases. Two groups failed to detect bcl-2 staining in RS cells (Pezzela *et al.*, 1990, Louie *et al.*, 1991); weak bcl-2 staining was detected in RS cells in five of nine HD cases (Zutter *et al.*, 1991) and Bhagat *et al.* (1993) detected bcl-2 protein in RS cells in eight of thirteen HD cases which had been previously analysed for t(14;18) translocation. In the latter study, the expression of bcl-2 in RS cells did not correlate with the presence of EBV LMP-1. Bhagat *et al.*, (1993) analysed the clinical features and histological subtype, EBV-positivity and t(14;18) translocation with bcl-2 expression and showed no association between any of these factors.

From the results of this and other studies, we conclude that the LMP-1 protein is present in approximately 40% of HD cases however, there is no evidence of EBNA-2 expression in HD tissues (Pallesen *et al.*, 1991a, Herbst *et al.*, 1991a). These results indicate that a distinct pattern of EBV latent gene expression exists for HD. Rowe *et al.* (1992) described three patterns of EBV latent gene expression, *in vitro* (section 1.4.3.6). In BL cell lines only EBNA-1 is detected (Rowe *et al.*, 1987). In LCL all of the latent proteins are observed (Young *et al.*, 1989). These latency patterns have been defined as latency (Lat) I and Lat III patterns, respectively. The pattern of expression in HD appears to be similar to that seen in NPC in which LMP-1 is expressed in the absence of EBNA-2 and is described as a Lat II expression pattern.

The role of LMP-1 in HD remains unresolved but the results of this study suggest that it is independent of EBNA-2 expression and the upregulation of CD23 and bcl-2. The demonstration of an EBV encoded protein with oncogenic potential expressed in the RS cells suggests that EBV plays a role in the pathogenesis of HD.

Chapter 4

Definition of EBV-association in Hodgkin's disease: a molecular analysis

4.1 Introduction

At the initiation of this work, the majority of studies had used Southern blot hybridisation to detect EBV in HD biopsy material (Weiss *et al.*, 1987a, Anagnostopolous *et al.*, 1989, Weiss *et al.*, 1989, Staal *et al.*, 1989, Bolocchi *et al.*, 1989, Gledhill *et al.*, 1991, Jarrett *et al.*, 1991a). The EBV BamHI-W probe, which identifies the major internal repeat sequence, can be used to detect a single EBV genome if it is present in <0.45% of a DNA sample derived from 1 x 10^6 cells (Jarrett *et al.*, 1991a). The sensitivity of this assay should enable the detection of EBV genomes present within RS cells in the majority of HD cases. In addition the examination of the terminal repeats of the viral genome using Southern blot analysis can be used to assess the clonality of the EBV-infected population (Raab-Traub & Flynn, 1986, see section 1.6.5). The EBV genomes in HD biopsies have been shown to be clonal which is consistent with the expansion of a single infected cell (Weiss *et al.*, 1987a, Bolocchi *et al.*, 1989, Jarrett *et al.*, 1991a).

Using DNA *in situ* hybridisation, EBV genomes had been detected within RS cells (Anagnostopolous *et al.*, 1989, Weiss *et al.*, 1989). However, these studies have been criticised due to a lack of sensitivity and poor signal-to-noise ratios. When applied to routinely fixed and processed material this technique often gives unsatisfactory results (Anagnostopolous *et al.*, 1989). Consequently, this is not a practical method for screening large case series and archival samples.

The use of the polymerase chain reaction (PCR) to detect EBV in large series of HD cases has the obvious advantages of ease and sensitivity. However, the sensitivity of this technique increases the likelihood of detecting EBV within the reactive infiltrate.

As described in the previous chapter, immunohistochemical studies have localised the EBV LMP-1 to RS cells (Pallesen *et al.*, 1991a, Armstrong *et al.*, 1992a, Brousset *et al.*, 1993b). As the EBV LMP-1 protein has been shown to have oncogenic potential (Wang *et al.*, 1985), these results indicated that EBV may be important in the disease process of HD. Further evidence to support this concept has been derived from the presence of EBV EBER RNA within RS cells (Wu *et al.*, 1990).

These small non-polyadenylated EBER RNAs (as described in detail in section 1.4.3.5), which are abundantly transcribed, are found within the nuclei of latently-infected cells (Howe & Steitz, 1986). Using an *in situ* hybridisation assay, Wu *et al.* (1990) reported the presence of EBER RNA within the RS cells in 6 of the 8 cases which were positive by Southern blot analysis.

In this study the optimal method of detecting EBV in HD for use in epidemiological studies was determined. The comparison of four methods, Southern blot analysis, PCR, immunohistochemical techniques and *in situ* hybridisation studies is described. The most practical method of determining the true incidence of EBV-association in HD is described.

4.2 Materials and Methods

4.2.1 Clinical samples

HD biopsies from a number of clinical centres were referred to the LRF Virus centre, University of Glasgow and examined for the presence of EBV. In total, 71 cases of HD were evaluated. Samples from 44 of the cases had been examined previously using Southern blot analysis and the EBV BamH1-W probe (Jarrett *et al.*, 1991a). Cases were selected on the basis of EBV status as assessed by Southern blot analyses and PCR and, where possible, these cases were investigated using immunohistochemical and *in situ* hybridisation methods.

4.2.1.1 Southern blot positive cases

Seventeen cases were EBV positive by Southern blot analysis. In sixteen of these cases we had demonstrated the clonality of the EBV genomes (Jarrett *et al.*, 1991a). The EBV-infected population in the remaining case (case 960) did not appear to be clonal (Jarrett *et al.*, 1991a). During the course of the present study, using a slightly altered strategy, the clonality of the EBV genomes in this case was demonstrated (Armstrong *et al.*, 1992b). In order to determine the cellular localisation of the EBV, paraffin-embedded tissue sections from these cases were analysed by immunohistochemistry (see Chapter 3) and *in situ* hybridisation.

4.2.1.2 Southern blot negative cases

Samples from 27 Southern blot negative cases were examined to determine whether EBV could be detected using more sensitive techniques. DNA samples

from 15 cases were examined for the presence of EBV genomes using PCR. The expression of LMP-1 protein and EBER-1 RNA was examined in 22 cases. As DNA samples from 12 cases had been handled in the laboratory in which plasmids containing EBV DNA fragments were used it was not possible to further analyse these cases by PCR because of the risk of contamination. Paraffin-embedded material from four cases was not available.

The association between expression of LMP-1, CD23 and bcl-2 in a proportion of these cases is reported in the previous chapter of this thesis.

4.2.1.3 Non-selected cases

Samples from 27 non-selected cases were examined for the expression of LMP-1 and EBER-1 in order to compare further the techniques used to detect these two EBV latent gene products.

4.2.2 Immunohistochemical analyses

EBV LMP-1 expression was investigated in paraffin-embedded HD sections using a cocktail of monoclonal antibodies (CS1-4) reactive with LMP-1 (Rowe *et al.*, 1987) as described in section 2.3.6 and 3.2.2.2-3.2.2.6.

4.2.3 RNA *in situ* hybridisation

Three to five micrometre paraffin-embedded sections were processed as described in section 2.2.3. All solutions were made up in diethyl pyrocarbonate (DEPC) treated water (Appendix V).

4.2.3.1 Pronase digestion

The proteolytic digestion of paraffin-embedded tissue is a critical step in the *in situ* hybridisation protocol. The digestion of paraffin-embedded lymph node tissue sections using pronase (Boehringer Mannheim UK, Lewes, UK) was tested at concentrations of 100μg/mL, 500μg/mL and 1mg/mL over the time periods two, five and ten minutes. Optimal proteolytic digestion of lymph node tissue using pronase was achieved at a concentration of 1mg/mL for five minutes at room temperature. These parameters varied depending on the proteolytic enzyme used and tissue type examined. Therefore for each tissue type examined the concentration of pronase and time of incubation were assessed. The proteolytic enzyme reaction was stopped by immersing the sections in glycine buffer (Appendix III). Sections were dehydrated by placing in 70%, 85% and 99.7% ethanol for five minutes each and air dried at room temperature.

4.2.3.2 Oligonucleotide probes

The *in situ* protocol was described by Weiss *et al.* (1990) and modified for the detection of EBV using an oligonucleotide probe specific for the EBER-1 sequence. An oligonucleotide probe complementary to the EBER-1 RNA was synthesised with a biotin molecule at the 5' end:

5'-AGA CAC CGT CCT CAC CAC CCG GGA CTT GTA-3'

Tissues infected with herpes simplex-1, human papilloma virus-16 and adenovirus did not show hybridisation with this oligonucleotide probe (Weiss *et al.*, 1991).

A nonsense oligonucleotide probe with base composition identical to that of the probe of interest was synthesised with a biotin molecule at the 5'-end.

5'-ATG GTG AGC CGG AGT CCT TAC GTC CGC GAA-3'

These probes were 3'-end labelled with biotin as described in section 2.4.2.

4.2.3.3 Hybridisation of oligonucleotide probe

The melting temperature (T_m) of the hybrid between the probe and target sequence is dependent on several factors; ionic strength, base composition and denaturing agents. These factors are all considered in the following formula:

$$T_m = 81.5 + 16.6(\log_{10}M) + 0.41\ (\%G+C) - 0.72\ (F) - 500/L$$

where T_m = temperature at which 50% of duplexes are dissociated

M = concentration of monovalent cation

%G + C = proportion of guanidine and cytidine nucleotides

F = % of formamide

L = length of duplex in base pairs

When calculating the optimal hybridisation temperature for RNA-DNA hybrids several factors are considered. The stability of RNA-DNA hybrids is greater in 50% formamide than that of a DNA duplex of similar base composition (Bishop, 1972). This allows RNA-DNA hybrids to form whilst preventing DNA duplex formation. The maximum rate of RNA-DNA hybridisation has been shown to be obtained at 10-15°C below the T_m of the hybrids. Thus using the above equation, the T_m of the EBER-1 oligonucleotide following hybridisation to a perfectly matched sequence is equal to 47.75°C under conditions of 50% formamide and 3x SSC (salt concentration, 0.45M). The optimal hybridisation temperature was calculated to be approximately 37°C for the EBV EBER-1 oligonucleotide probe.

The sections were incubated with pre-hybridisation buffer (Appendix III) for two hours at 37°C. The probe was tested at a range of probe concentrations between

0.125-10ng/μL. A final probe concentration of 0.25ng/μL was optimal and this was diluted in hybridisation buffer (Appendix III). The sections were subsequently hybridised for 16-20 hours at 37°C .

4.2.3.4 Detection of biotinylated probes

All post-hybridisation wash steps were carried out at room temperature. The detection of biotinylated oligonucleotide probes was carried out as described in section 2.4.4.

4.2.3.5 Controls

A series of controls were included in each of the *in situ* hybridisation assays. In each assay an EBV-positive HD-derived cell line, L591, and the T-cell line, J-Jhan, embedded in paraffin wax, were included as positive and negative controls, respectively. In some studies these controls were not available thus a positive case identified during previous experiments was included as a positive control. The nonsense oligonucleotide probe (see section 4.2.3.2) was included during the optimisation of the assay on paraffin-embedded tissue from different clinical centres in order to assess non-specific staining. In order to determine the integrity of RNA in the tissue samples some negative cases were probed with a 30 base pair oligonucleotide dT probe, using altered hybridisation conditions: 25% formamide hybridisation buffer and 3x SSC at 37°C.

4.2.4 PCR analyses

DNA samples were assayed using primers derived from the EBV BamH1-W sequence (Deacon *et al.*, 1991), the EBNA-2 gene (Sample *et al.*, 1990) and the LMP-1 gene (Brooks *et al.*, 1992) (Table 4.1). The EBV genome can be subtyped into Types 1 or 2 using the EBNA-2 primers (Sample *et al.*, 1990).

A standard polymerase chain reaction was performed. Two micrograms of DNA were assayed in a 50μL total reaction mixture containing 1μM primers (50pmol), 1.5mM magnesium chloride, 50mM potassium chloride, 10mM Tris pH 8.2, 0.05% NP40, 200μmol/L of each deoxynucleotide triphosphate, and 1U of Amplitaq (Perkin-Elmer, Applied Biosystems, Warrington, UK). The reaction mixture was overlaid with mineral oil to prevent evaporation during thermal cycling. Initial denaturation of target DNA was carried out at 95°C for seven minutes in a thermal cycling machine (Perkin-Elmer Instruments). Following a ramp time of one minute, primers were denatured at 94°C for 10 seconds. The temperature was reduced to 55°C over two minutes and the primers were annealed at this temperature for 10 seconds. The temperature was increased to 72°C over one minute and held at this temperature for 30 seconds to allow extension of the annealed primers using Taq polymerase. Forty cycles of amplification were performed. An extension time at 72°C for 7 minutes was performed and the samples were finally brought to a temperature of 6°C.

DNA from the EBV-positive cell line B95-8 and three Southern blot positive HD samples were used as positive controls. A negative control, consisting of either water or placental DNA, was included after each sample. Identical aliquots of the

Table 4.1 Primer and probe selection for the polymerase chain reaction

	Primer or probe sequence	Size of product
Primers		
β-globin Saiki *et al.*, 1988	5'-ACA CAA CTG TGT TCA CTA GC-3' 5'-CAA CTT CAT CCA CGT TCA CC-3'	110bp
EBV BamHI-W Deacon *et al.*, 1991	5'-TGA CTT CAC CAA AGG TCA GG-3' 5'-AGG ACC ACT TTA TAC CAG GG-3'	140bp
EBNA-2A Sample *et al.*, 1990	5'TTG TGA CAG AGG TGA CAA AA-3' 5'-AGG GAT GCC TGG ACA CAA CA-3'	249bp
EBNA-2B Sample *et al.*, 1990	5'-TTG AAG AGT ATG TCC TAA GG-3' 5'-AGG GAT GCC TGG ACA CAA GA-3'	300bp
EBV LMP-1 Brooks *et al.*, 1992 (nested primers)	5'-CTT CAG AAG AGA CCT TCT CT-3' 5'-ACA ATG CCT GTC CGT GCA AA-3'	182bp
Probes		
β-globin	5'-CTC CTG AGG AGA CTG C-3'	
EBV Bam H1-W	5'-AGC GCG TTT ACG TAA GCC AGA CAG CAG CCA ATT GTC ACT T-3'	
EBNA-2A	5'-TCC AGC CAC ATG TCC CCC CTC TAC GCC CGA CA-3'	
EBNA-2B	5'-AGG GTC AAC CTG TCC ACA ACC CTC GCC AGG AG-3'	

DNA samples were tested using the β-globin primers (Table 4.1), in order to check that amplifiable DNA was present.

Ten microlitres of the amplified products were subjected to polyacrylamide gel electrophoresis, electroblotted onto nylon membranes and hybridised with specific probes (Table 4.1). These assays were performed by Alice Gallagher.

4.3 Results

The RS cells within a proportion of HD cases showed reactivity with the CS1-4 antibodies and hybridised with the EBER-1 probe (Tables 4.2 and 4.3). The pattern of labelling with the CS1-4 antibodies was predominantly cytoplasmic, but occasional cells showed membrane positivity (Figure 4.1). As illustrated in Figures 4.2a and 4.3, the EBER-1 RNA was detected within the nuclei of positive cells. No specific staining was detected in the tissue sections following hybridisation with the nonsense (control) oligonucleotide probe (Figure 4.2b). Hybridisation with the EBER-1 probe was not restricted to RS cells as staining of small lymphocytes was also observed (Figure 4.4).

4.3.1 Southern blot positive cases

In 13 of the 17 cases in which clonal EBV genomes had been detected, RS cells were stained by the CS1-4 antibody cocktail. RS cells in three of the remaining four cases hybridised with the EBER-1 oligonucleotide probe. The cellular localisation of EBV was not established in case 962 and further investigation showed that sections from this case did not hybridise with an oligonucleotide dT probe. These results suggested that the RNA was degraded. The majority of these cases had been subtyped into EBV types 1 and 2 using a PCR strategy (Tables 4.2 and 4.3) (Jarrett *et al.*, 1991a).

4.3.2 Southern blot negative cases

PCR analyses were performed on 15 samples within this group and positive results were obtained in 13 cases (Table 4.3). Quantitative PCR was not performed but

Table 4.2 Detection of EBV in selected Hodgkin's disease cases

Southern blot positive HD cases

LRF case number	Histological subtype	Age (years)	EBV subtype	*LMP-1	*EBER-1
70	LP	36	1	+	+
79	MC	48	1	+	+
386	MC	80	1	+	+
387	MC	14	1	+	+
390	NS	68	1	+	ND
937	MC	14	1	+	+
938	NS	6	ND	+	+
940	MC	5	1	+	+
954	MC	31	1	+	+
960	NS	59	ND	-	+
962	NS	53	1	-	-
974	NS	82	1	+	+
981	MC	85	1	+	+
982	MC	78	1	-	+
987	LP	65	1	-	+
990	MC	67	1	+	+
998	MC	7	1	+	+

NS, nodular sclerosis HD; MC, mixed cellularity HD; LP, lymphocyte predominance HD.

+. positive result; - negative result; ND not done; * EBV detected in RS cells

Table 4.3 Detection of EBV in selected Hodgkin's disease cases

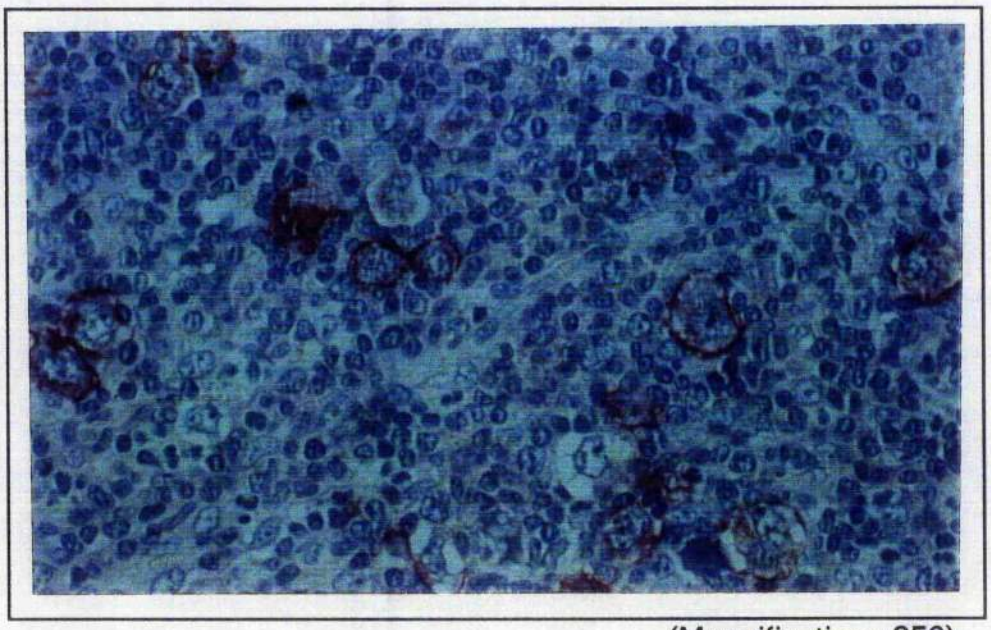
Southern blot negative HD cases

LRF case number	Histological subtype	Age	PCR	EBV subtype	LMP-1	EBER-1
71	NS	22	+	1	-	-
74	MC	20	++	-	+	+
75	NS	24	++	1	+	+
420	LD	19	+	1	-	-
430	NS	67	++	2	-	-
924	NS	19	+	1	-	-
926	NS	26	++	1	-	-
382	NS	23	+	1	-	-
384	NS	28	+	1	ND	-
385	NS	31	+	-	-	-
929	LP	17	-	-	ND	ND
930	NS	32	-	-	ND	ND
931	NS	42	+	1	-	-
932	NS	25	++	1	ND	ND
934	NS	21	+	1	ND	ND
939	NS	7	ND	ND	-	-
951	MC	21	ND	ND	-	-
955	MC	24	ND	ND	-	-
956	MC	25	ND	ND	-	+
959	MC	19	ND	ND	-	-
963	NS	54	ND	ND	-	-
964	NS	56	ND	ND	-	-
979	MC	61	ND	ND	-	-
983	MC	60	ND	ND	-	-
984	MC	82	ND	ND	-	-
995	NS	39	ND	ND	-	-
999	NS	18	ND	ND	-	-

NS, nodular sclerosis HD; MC mixed cellularity HD; LP, lymphocyte predominance HD; LD, lymphocyte depleted HD.

+, positive result; ++, positive result on ethidium-bromide stained gels; -, negative result; ND not done. *EBER-1 detected in RS cells

Figure 4.1 Expression of EBV LMP-1 in HD Case 390



(Magnification x250)

Paraffin-embedded lymph node section from a 68 year old NSHD case. Reactivity with the LMP-1 monoclonal antibody (CS1-4) is detected in RS cells, multinuclear cells and mononuclear variants. EBV genomes were clonal. Alkaline phosphatase and Fast red salt, counterstained with haematoxylin.

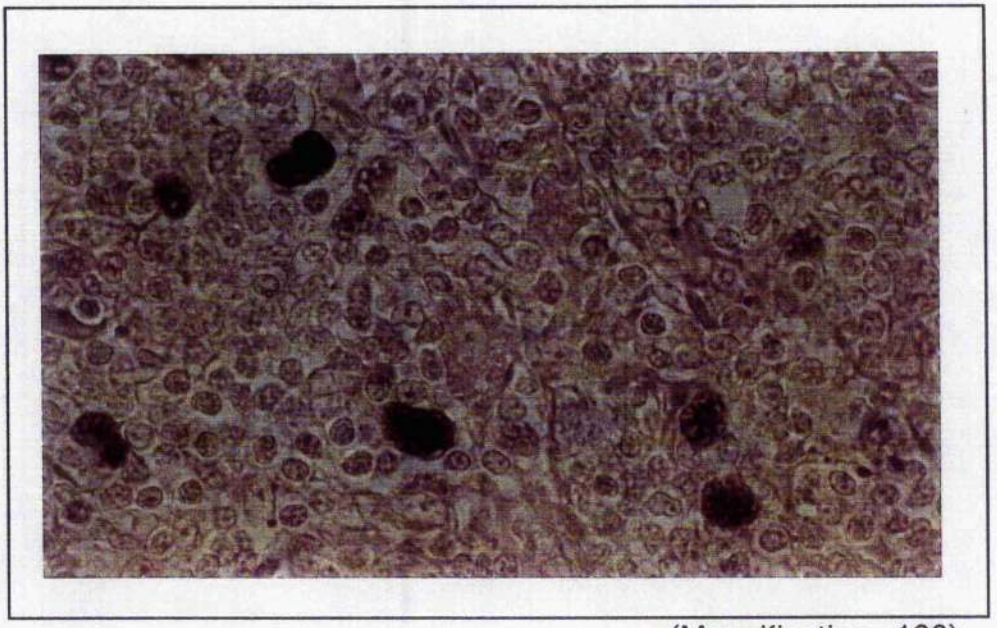
Figure 4.2a: EBER RNA *in situ* hybridisation: Case 981

Paraffin-embedded lymph node section from a 85 year old MCHD case. Clonal EBV genomes were detected. EBV LMP-1 was not expressed. EBER RNA is detected in RS cells. Note nuclear localisation of EBER RNA. Alkaline phosphatase and NBT, lightly counterstained with haematoxylin.

Figure 4.2b: *In situ* hybridisation using nonsense oligonucleotide probe as negative control: case 981

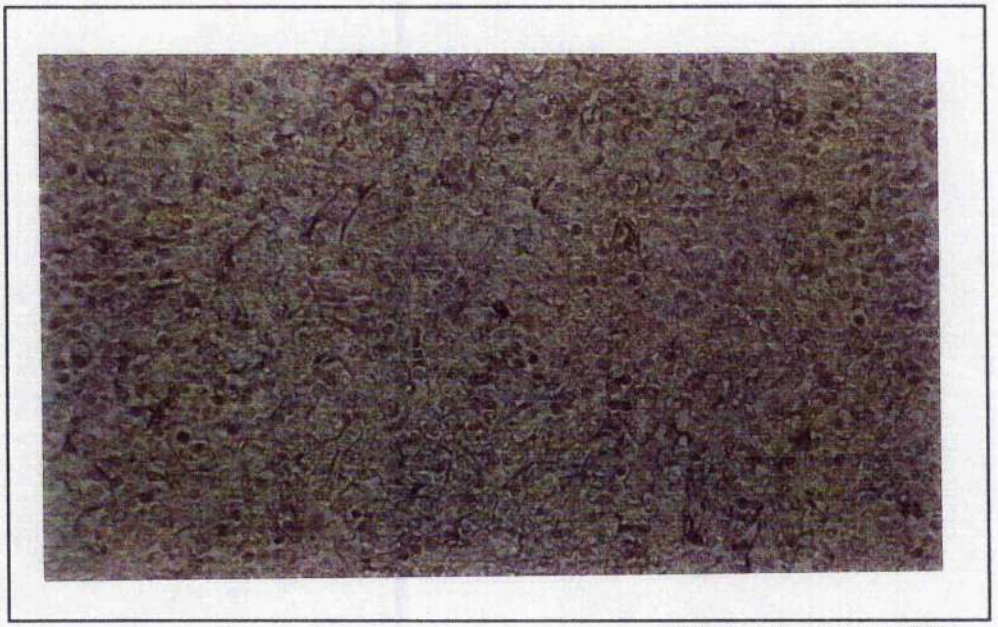
Paraffin-embedded tissue section from a 85 year old MCHD case. Hybridisation of biotin-labelled nonsense oligonucleotide probe as negative control. Alkaline phosphatase and NBT not counterstained.

Figure 4.2a EBER RNA *in situ* hybridisation
Case 981



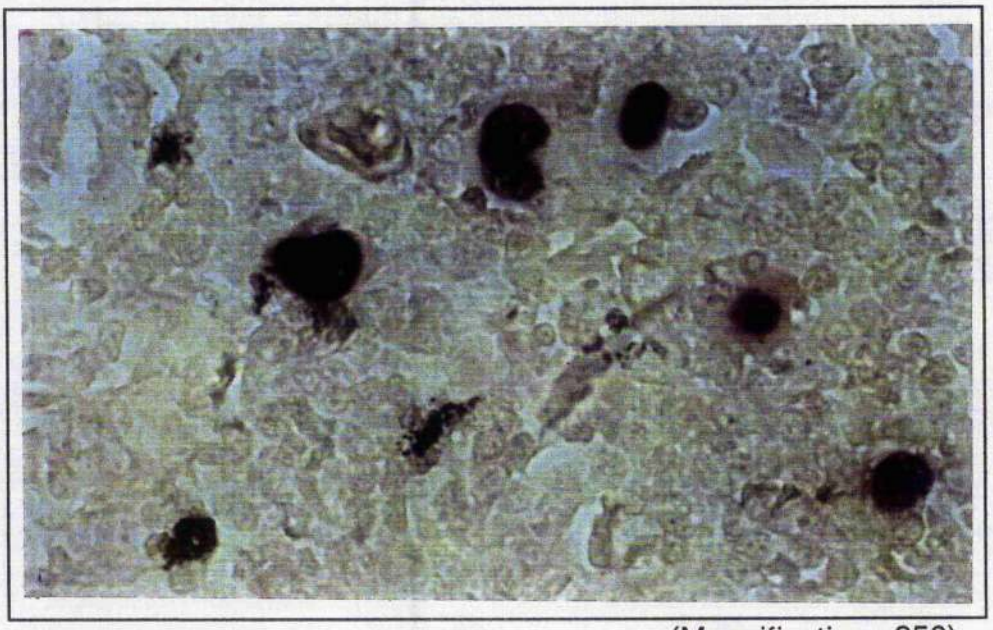
(Magnification x100)

Figure 4.2b *In situ* hybridsation using nonsense oligonucleotide probe as negative control
Case 987



(Magnification x 50)

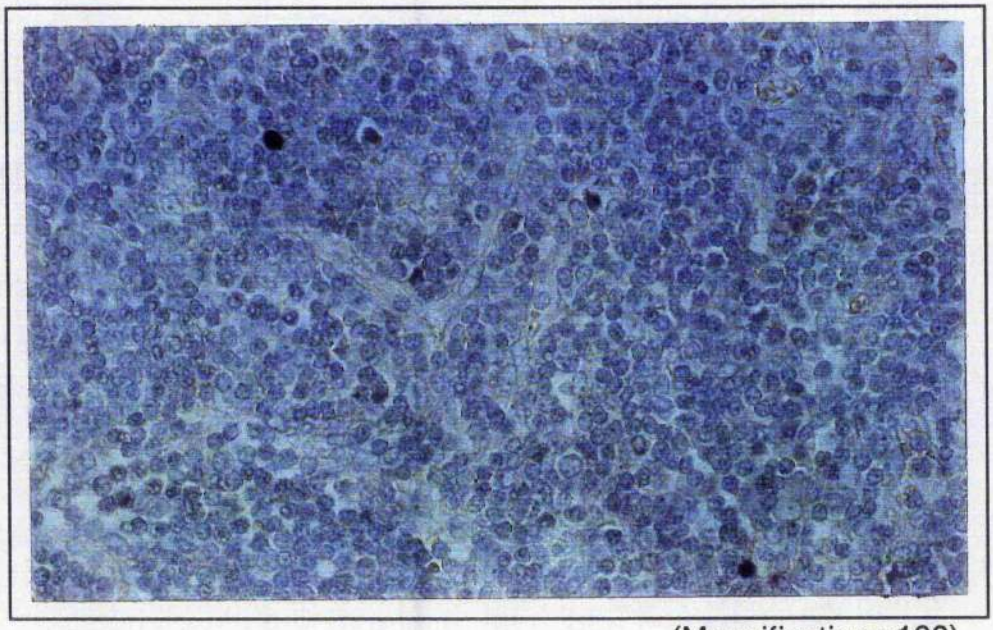
Figure 4.3 EBER RNA *in situ* hybridisation
Case 982



(Magnification x250)

Paraffin-embedded lymph node section from a 78 year old MCHD case. EBER RNA is detected in RS cells. Clonal EBV genomes were detected. LMP-1 was not detected in RS cells. Alkaline phosphatase and NBT, not counterstained.

Figure 4.4 EBER RNA *in situ* hybridisation
Case 926



(Magnification x100)

Paraffin-embedded lymph node section from a 26 year old NSHD case. EBV-positive by PCR on ethidium bromide stained PAGE gels. EBV-negative by Southern blot hybridisation. LMP-1 was not detected. Note staining of small lymphocytes. Alkaline phosphatase and NBT, counterstained with haematoxylin.

samples were scored as strongly positive (++) if specific products were detectable on ethidium bromide-stained gels and positive (+) if hybridisation were required. Five of the above samples scored (++). Previous experimental procedures have concluded that samples which are positive for EBV on Southern blot analysis are usually strongly positive using PCR.

As expected, the assay using primers derived from the EBV BamHI-W sequence, the viral internal repeat, was most sensitive but there was good concordance between the results of the three PCR assays. One sample (case 385) was positive in only the assay using BamH1-W primers. DNA from case 74 did not amplify with EBNA-2 primers and the viral genome in this case is under further investigation in order to determine whether it is defective. Of the eleven samples which were successfully subtyped using EBNA-2 primers, ten were subtype 1 and the remaining case (case 430) was subtype 2.

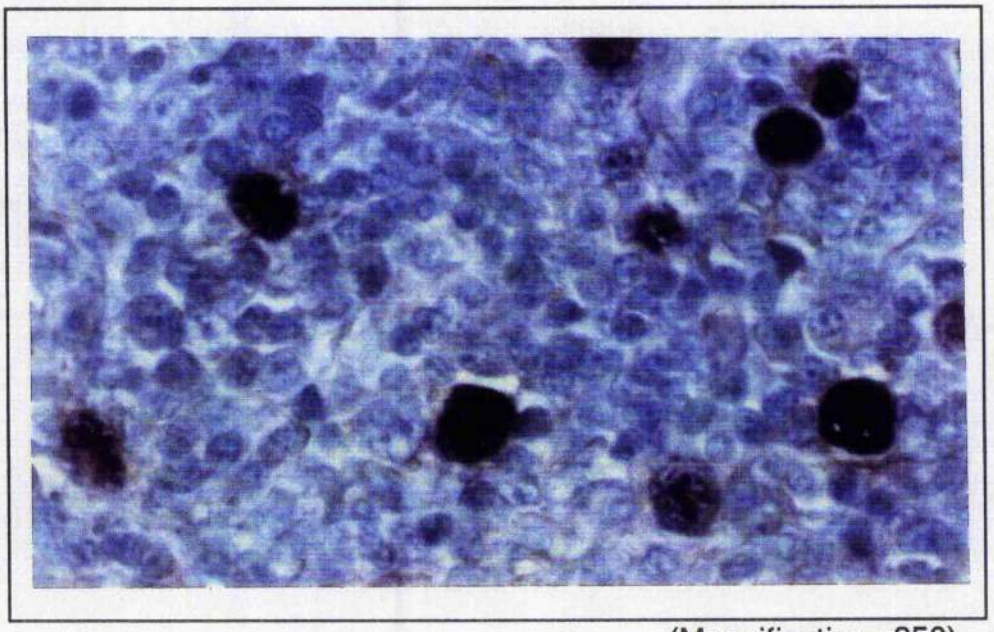
Immunohistochemical staining for LMP-1 was performed on sections from 22 cases and in two cases (cases 74 and 75) the RS cells were stained by the CS1-4 antibody cocktail (see Chapter 3, Figures 3.4a and 3.5). Expression of EBER-1 RNA was detected in RS cells within sections from these 2 cases and from a third case in this group (case 956). The latter case was originally scored as negative in the immunohistochemical analysis, but on histological review a few abnormal cells showed weak reactivity with the CS1-4 antibodies.

In two cases (cases 430 and 926, see Figure 4.4), scattered lymphocytes hybridised with the EBER-1 probe.

4.3.3 Non-selected cases

From the results of the above analysis the EBER-1 RNA *in situ* hybridisation analysis appeared to be the preferred method for evaluating the EBV status of HD tumours. The immunohistochemical and *in situ* hybridisation methods were compared in a fresh series of 27 cases. The RS cells within nine cases showed expression of EBV latent gene products; eight cases reacted with the LMP-1 antibodies and in eight cases the RS cells hybridised with the EBER-1 oligonucleotide probe (Figure 4.5). In samples positive by both assays the number of cells showing hybridisation with the EBER-1 probe was greater than that stained with the antibodies to LMP-1.

Figure 4.5 EBER RNA *in situ* hybridisation
Case 1221



(Magnification x250)

Paraffin-embedded lymph node section from a non-selected 25 year old MCHD case. Note detection of EBER RNA in RS cells. Using an immunohistochemical method, LMP-1 was detected in RS cells. Alkaline phosphatase and NBT, counterstained with haematoxylin.

4.4 Conclusions

This study evaluates the assays which have been used to detect EBV within biopsy samples from HD patients. In agreement with previous studies, the EBV latent gene products EBER-1 and LMP-1 were detected in RS cells (Pallesen *et al.*, 1991a, Weiss *et al.*, 1991, Murray *et al.*, 1992, Brousset *et al.*, 1993b). Although the function of the EBER RNAs is not fully understood (see section 1.4.3.5), the high abundance and expression of these RNAs during latent infection provides a useful tool for localising EBV to RS cells in HD.

From this study, EBV gene products were detected within RS cells of only 3/23 cases which were negative on Southern blot analysis. However, EBV was localised to RS cells in only 2/13 Southern blot negative, PCR positive samples. In many of the remaining cases scattered small lymphocytes within the tissue sections hybridised with the EBER-1 probe. In previous studies, the use of PCR to detect EBV genomes in HD greatly increased the proportion of EBV-positive cases identified. EBV-positivity rates of between 18-79% were observed in HD biopsy material following PCR analysis (Uhara *et al.*, 1990, Herbst *et al.*, 1990, Wright *et al.*, 1991, Weiss *et al.*, 1991, Knecht *et al.*, 1991, Masih *et al.*, 1991, Brocksmith *et al.*, 1991). Therefore, PCR analysis alone may lead to an over-estimation of the number of cases in which EBV is involved.

The results of this study and other studies support the suggestion that EBV is present within small lymphocytes and was responsible for the increased numbers of EBV-positive HD cases as shown by PCR analysis (Weiss *et al.*, 1991, Brousset *et al.*, 1993b). The significance of these EBV-positive small reactive lymphocytes in the pathogenesis of HD is not known. However the presence of these cells may be

a consequence of immune dysfunction in HD patients (Slivnick *et al.*, 1990). Thus PCR may only prove useful if performed quantitatively and in combination with localisation assays. In addition, a number of variables including the number of RS cells within a biopsy and the viral copy number must be taken into account during the interpretation of these assays.

Overall these data suggest that there is a relationship between the amount of EBV DNA present in the tumour and the cellular localisation of the EBV. Southern blot analysis has a good positive predictive value for the presence of EBV in RS cells, but is not sufficiently sensitive to detect EBV in all cases.

EBV is likely to play a direct role in the pathogenesis of HD in those cases in which the RS cells represent clonal expansions of an EBV-infected cell. Two assays, the immunohistochemical analysis and *in situ* hybridisation, permit localisation of EBV to the abnormal cell population. There is a good correlation between the presence of EBER and LMP-1 expression in RS cells (reviewed by Drexler, 1992, Hummel *et al.*, 1992, Brousset *et al.*, 1993b). In addition, Southern blot analysis permits demonstration of clonality and has a good correlation with the *in situ* techniques. Samples from 37 cases were analysed by all three methods and 19 cases were scored as positive in at least one assay. The EBER-1 *in situ* hybridisation assay was positive in 18 of these cases, clonal EBV genomes were demonstrable in 16 cases by Southern blot analysis and the immunohistochemical assay was positive in 15 cases. Thus, the RNA *in situ* hybridisation assay appears to be the most sensitive method for detecting EBV present in RS cells. The relative stability of EBER-1 RNA renders this assay suitable for analysis of fixed material. At present, this is the optimal way to detect EBV within HD lesions.

HD tumours, therefore, can be subdivided into three groups on the basis of their EBV status. Due to the selected nature of the cases included in the study the exact proportion of cases in each group can only be estimated. In the first group, which comprises 33-50% cases, the EBV is present and expressed in RS cells. In the majority of these cases clonality of the EBV-infected cell population can be demonstrated. The EBV in this group of cases is likely to play a role in the disease process. We have designated as *EBV-associated* those cases in which clonal EBV genomes are detected and the EBV LMP-1 protein or EBER RNA are present in RS cells.

In the second group, which included approximately half of the cases, EBV is detectable using sensitive techniques but appears to be present in small lymphocytes. The distinction between localisation of EBV to the malignant cells and to the small lymphocytes is clearly necessary. The RNA *in situ* hybridisation method enables this distinction to be made.

EBV was not detectable in HD biopsy material in the third group which comprises only a small minority of cases.

Future studies of the pathogenesis of HD should take into account the EBV status of the lesions. The use of the above mentioned criteria to define EBV-associated HD cases should overcome confusion in the literature concerning the definition of an EBV-positive case.

Chapter 5

Detection of EBV in paediatric Hodgkin's disease

5.1 Introduction

Although HD is not common the high incidence of cases in young adulthood has generated a great deal of interest in both the biological and clinical aspects of this malignancy.

HD has a bimodal age incidence curve. In developed countries, HD is rare in childhood, however there is an increased incidence in young adults (Cartwright *et al.*, 1990). In developing countries higher rates of HD are observed in childhood and there is no adult peak (Correa & O'Conor, 1971). An intermediate age incidence pattern has been observed in countries undergoing socio-economic change (Correa & O' Conor, 1971, Alexander *et al.*, 1991b). In both developed and developing countries the incidence of HD increases or plateaus in older age groups (McKinney *et al.*, 1989). The patterns of age incidence in HD are described in more detail in section 1.3.4.

Examination of the age incidence data and other epidemiological features of HD led MacMahon (1966) to suggest that this disease comprises of three distinct disease entities, based on the age at clinical onset. HD in young adults was thought to be caused by an infectious agent. There is little additional data available for the paediatric age group (Gutensohn, 1982, Alexander *et al.*, 1991a).

Studies investigating the epidemiological features of HD have resulted in the suggestion that young adult cases of HD occur as a host response to late primary exposure to a common infectious agent (Alexander *et al.*, 1991a, Gutensohn & Cole, 1980). Similarity between the epidemiological features of HD and paralytic poliomyelitis-the *polio model* has been observed (Gutensohn & Cole, 1980). In

contrast to MacMahon's hypothesis, this model suggests that the same infectious agent is involved in young adult and childhood HD (see section 1.3.4).

These two hypotheses are consistent with HD in young adults being related to late exposure to a common infectious agent. Evidence to support an infectious aetiology for HD has been accumulating over a number of years. As demonstrated in the previous chapter of this thesis HD cases can now be designated as either *EBV-associated* of non-associated based on the presence of EBV within RS cells. Using sensitive and reproducible *in situ* hybridisation methodology the localisation of EBV within RS cells in formalin-fixed HD tissues has enabled the study of increased numbers of cases from different age groups.

Only a limited number of paediatric HD cases have been studied previously using Southern blot hybridisation. In the present study a number of paediatric HD cases from the United Kingdom (UK) were examined for the presence of EBV. Cases of paediatric HD from Brazil and Saudi Arabia were also investigated.

The differences in EBV-positivity rates for Saudi Arabia and Brazil were compared with those of the UK cases. The results of EBV status of the cases from all three countries were statistically analysed with respect to age, sex and histological subtype. In order to study the *polio model* and MacMahon's hypothesis in relation to EBV, the proportion of EBV-associated cases of paediatric and young adult HD cases from the UK was compared.

5.2 Materials and Methods

5.2.1 Clinical samples

Tumour biopsy samples from HD cases under the age of 15 years were obtained from a number of pathological centres throughout the United Kingdom. In addition formalin fixed paraffin-embedded tissue sections from paediatric HD cases were received from Dr R. Pinto Paes, Patologia, Sao Paulo, Brazil and Dr N.A. Morad, King Saud University, Abha, Saudi Arabia.

In total 55 paediatric HD cases selected by country (18 females: 37 males) were included in this study. These included 22 cases from the UK, 25 cases from Sao Paulo, Brazil and 8 cases from Saudi Arabia. All of the cases from Brazil and Saudi Arabia were native to those countries, whereas one of the cases from the UK was of Asian origin. With the exception of a single UK case, from which no paraffin-embedded material was available, all cases were reviewed by Dr A.S. Krajewski, Consultant Pathologist, Edinburgh Royal Infirmary, Edinburgh. One case was considered to have a differential diagnosis of LDHD and anaplastic large cell lymphoma but was retained in the study. The cases were classified using the Rye classification system (Lukes *et al.*, 1966); 24 NSHD, 24 MCHD cases, 4 LPHD and 3 LDHD.

The cases from Brazil were all HIV-negative, however HIV testing was not carried out on the remaining cases for ethical reasons.

The features of the Sao Paulo region of Brazil suggested that this area may be representative of a developing country; the incidence of paediatric HD in males is

highest in the age group 5 to 9 years, the incidence of acute lymphoblastic leukaemia (ALL) is low, and a high incidence of non-Hodgkin's lymphoma (NHL) in young children is observed (Parkin *et al.*, 1988). In Saudi Arabia, no cancer registry exists but the features of ALL are similar to those described for Western countries (Roberts *et al.*, 1990).

5.2.2 Classification as EBV-associated or non-associated HD

The comparison of several different methods for detecting EBV in HD was reported in Chapter 4. From these data the optimal method of detecting EBV in HD was found to be EBER-1 RNA *in situ* hybridisation. All cases from Saudi Arabia and Brazil were examined using this assay. Several methods were used to analyse the UK paediatric HD cases and these are summarised in Table 5.1.

Fourteen of the UK cases were examined for the presence of EBV using Southern blot analysis and 13 were included in a previous report (Jarrett *et al.*, 1991a). In the present study 13 of these cases were investigated further using *in situ* hybridisation. Paraffin-embedded material was not available from the remaining case, however Southern blot analysis indicated that this case was EBV-positive. One of the additional 9 UK cases was investigated for the presence of EBV using immunohistochemical staining for the detection of EBV LMP-1 protein. *In situ* hybridisation was not carried out due to the lack of available material. Fifty three of the cases including all cases from Saudi Arabia and Brazil were examined using EBER *in situ* hybridisation.

Table 5.1 Results of EBV analyses-UK paediatric HD cases

LRF case number	Age (years)	Histological subtype	Southern blot result	LMP-1*	EBER-1*
387	14	MC	C	+	+
698	7	MC	C	+	+
702	10	NS	NT	+	Z
927	13	NS	C	NT	NT
936	5	MC	+	NT	+
937	14	MC	C	+	+
938	6	NS	C	+	+
939	7	NS	-	-	-
940	5	MC	C	+	+
941	13	MC	-	+	+
942	12	LD	+	+	+
943	14	NS	-	-	-
944	14	NS	-	-	-
945	13	LP	-	-	-
946	10	NS	-	-	-
1092	11	NS	NT	-	-
1178	11	NS	NT	-	NT
1185	7	NS	NT	+	+
1220	4	MC	NT	+	+
1227	13	LD	NT	+	+
1235	13	NS	NT	-	-
1236	14	NS	NT	-	-

NS, nodular sclerosis HD; MC, mixed cellularity HD; LD, lymphocyte depleted HD; LP, lymphocyte predominance HD

C, clonal EBV genomes; Z, tissue section not satisfactory, NT, not tested; +, positive; -, negative; * EBV within RS cells

5.2.3 Detection of EBV

All *in situ* investigations were carried out on formalin-fixed paraffin-embedded material.

5.2.3.1 *In situ* hybridisation

Using methodology described in sections 2.4 and 4.2.3, paraffin-embedded biopsy sections from HD cases were examined for the presence of EBV EBER RNA, which is abundantly transcribed in cells latently infected with EBV.

5.2.3.2 Immunohistochemical analysis

The expression of EBV LMP-1 was examined using a cocktail of monoclonal antibodies (CS1-4) reactive with the LMP-1 protein and an ABC method as described in section 2.3.6 and in more detail in section 3.2.2.

5.2.4 Statistical methods

The statistical analysis was carried out by Dr F.E. Alexander, Edinburgh University Medical School, Edinburgh. The statistical package EGRET was used throughout. Equality of proportions in single tables was tested using Fisher's exact test. In order to adjust for the influence of other factors (e.g. age and histological subtype) the tables were stratified and more general tests applied.

5.3 Results

5.3.1 Detection of EBV in paediatric HD

In total 38 of the 55 paediatric cases were shown to be EBV-associated. The results are summarised in Table 5.2. EBV was detected in the RS cells in 35/53 cases analysed by EBV EBER-1 *in situ* hybridisation. These results included 7/8 and 18/25 cases from Saudi Arabia and Brazil, respectively. A high percentage of the RS cells in the biopsy tissue from both Brazil and Saudi Arabia stained positively as demonstrated in Figures 5.1 and 5.2. In the remaining cases scattered lymphocytes stained positively in the EBV EBER-1 *in situ* hybridisation assay.

Thirteen of the 22 UK cases, including the case with the differential diagnosis of anaplastic large cell lymphoma, were EBV positive. One case which was examined solely by immunohistochemical staining was positive and, as stated above the case which was examined by Southern blot analysis only was EBV positive. EBER-1 expression in RS cells was detected in a single case which was scored as negative previously on the basis of Southern blot analysis.

5.3.2 EBV-association by country

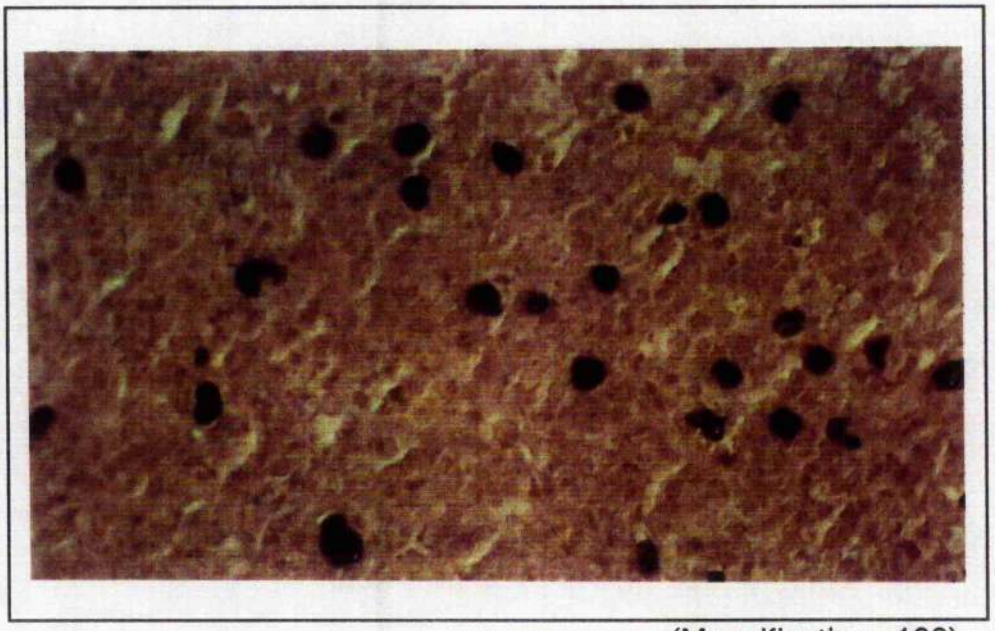
The majority of cases from each of the three countries were EBV-associated (Figure 5.3). The proportion of EBV-associated cases was higher in Saudi Arabia and Brazil than in the UK but differences between the three countries are not statistically significant ($p=0.34$ by Fisher's exact test). The cases from Brazil were younger than those from the UK (15/25 and 8/22, respectively being aged <10 years) and this was reflected in increased proportions of the HDMC subtype. The unstratified odds

Table 5.2 EBV-association and histological subtype by country in paediatric HD

HD subtype	UK	Brazil	Saudi Arabia	Total
NSHD	4/12	7/10	2/2	13/24
MCHD	7/7	10/12	5/5	22/24
LPHD	0/1	0/2	0/1	0/4
LDHD	2/2	1/1	0/0	3/3
Total	13/22	18/25	7/8	38/55

NSHD, nodular sclerosis HD; MCHD, mixed cellularity HD; LPHD, lymphocyte predominance HD; LDHD, lymphocyte depleted HD

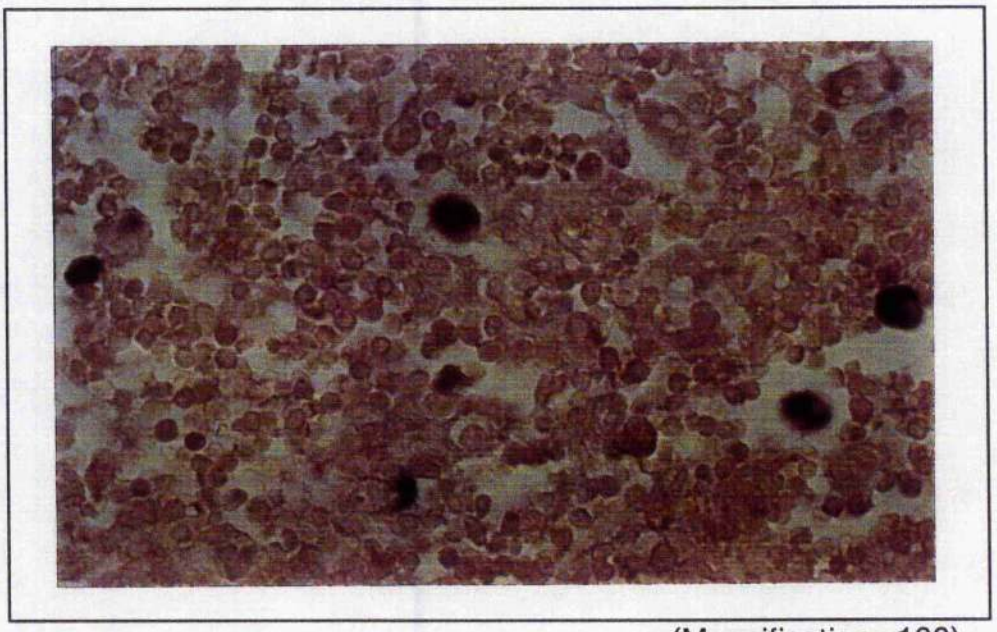
Figure 5.1 EBER RNA *in situ* hybridisation
Case 1386



(Magnification x100)

Paraffin-embedded lymph node section from a 5 year old paediatric MCHD case from Saudi Arabia. EBER-1 RNA detected in RS cells. Note distinct nuclear localisation of EBER RNA. Alkaline phosphatase and NBT

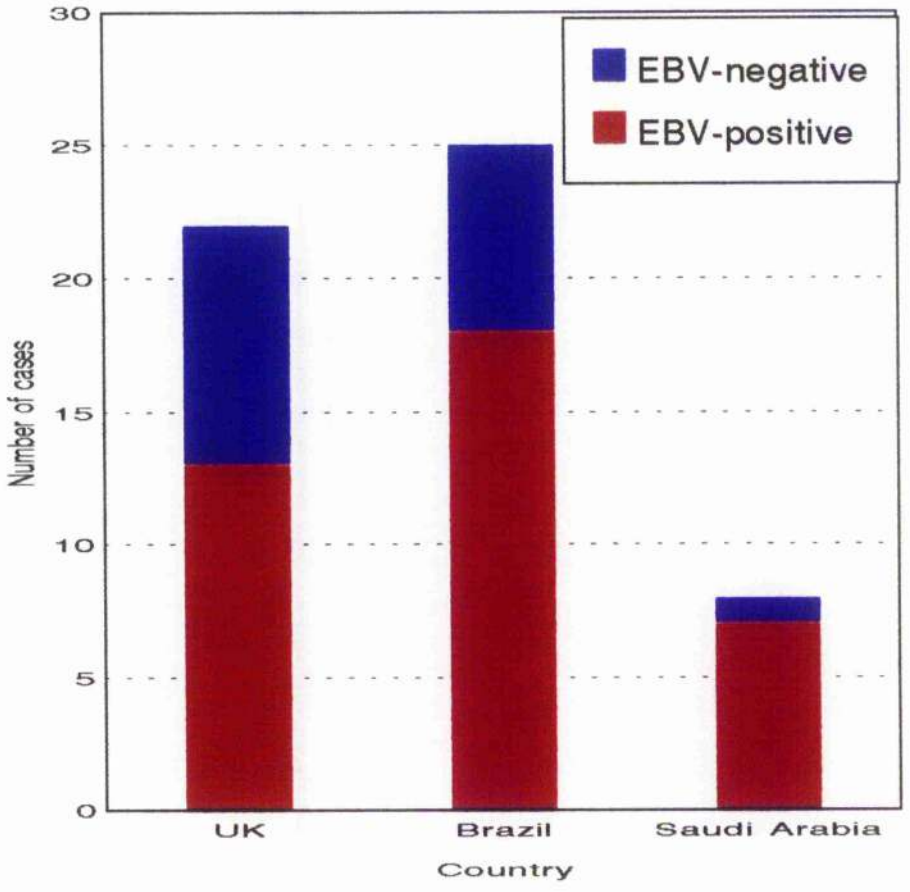
Figure 5.2 EBER RNA *in situ* hybridisation
Case 2050



(Magnification x100)

Paraffin-embedded lymph node section from a 4 year old paediatric MCHD case from Brazil. EBER RNA is detected in RS cells. Note distinct nuclear localisation of EBER RNA. Alkaline phosphatase and NBT, not counterstained.

Figure 5.3 EBV-association by country
Paediatric HD cases



ratios (OR) for EBV-association in Brazil cases (OR=1.54; 95% confidence limits=0.40-6.04) relative to the UK decreased to less than one when the data were adjusted for age and/or sex and/or histological subtype. These results show no evidence that children in Brazil are more likely to be EBV positive than UK cases if their age and sex are taken into account.

5.3.3 EBV-association by age

Cases were categorised into three age groups for this analysis, 1-4 years, 5-9 years and 10-14 years. In the youngest group 6/7 cases were EBV-associated; the single negative case was a 3 year old HDLP case from Saudi Arabia. In the 5-9 year old group 18/20 cases were EBV-associated, this decreased to 14/28 cases in the older group. This data is summarised in Figure 5.4. The effect of age on EBV-association between the age ranges 0-9 years and 10-14 years was tested (OR=0.12 for the 10-14 year age; 95% confidence limits=0.20-0.53, p=0.002). These results persisted when the data were stratified by sex or country or subtype (p=0.003). The differences in EBV association by age group are highly significant (p=0.004 by Fisher's exact test). This pattern was evident for the UK and Brazil cases when separate analyses were performed. Numbers were too small to permit a similar analysis of the cases from Saudi Arabia however the only negative case from Saudi Arabia was in the youngest group as mentioned above

The difference in EBV-association rate between the paediatric cases and those of a series of young adult HD (15-24 years) cases from the UK, in which 11/79 (13.9%) cases were EBV-positive, is highly statistically significant (p<0.001; Figure 5.5).

Figure 5.4 EBV-association by age

Paediatric HD cases

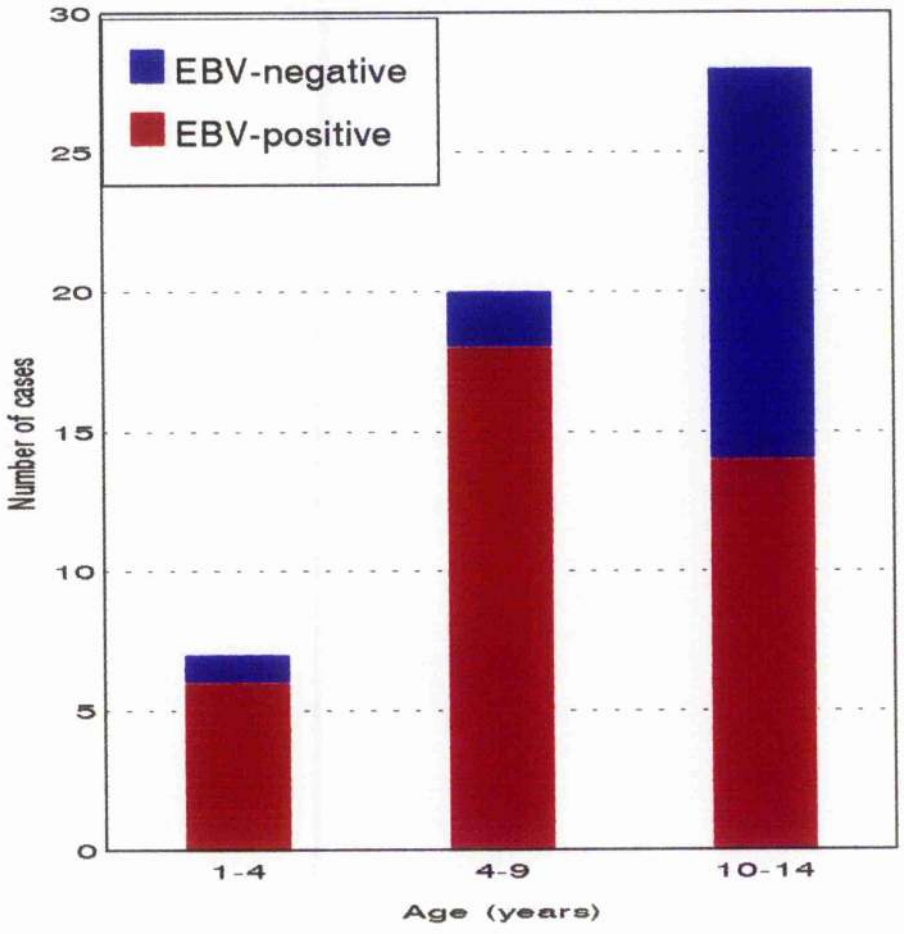
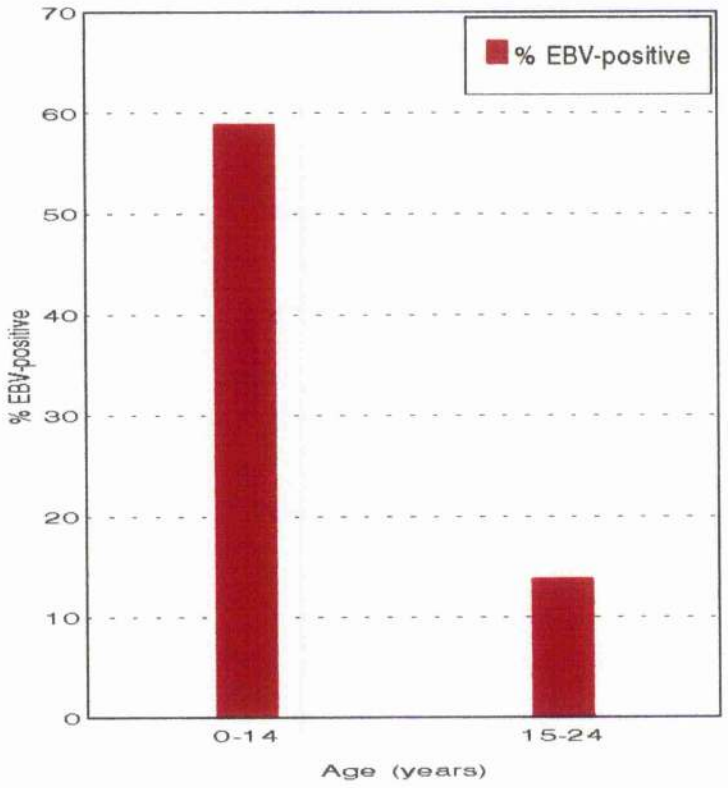


Figure 5.5 EBV-association by age

Testing the polio model in relation to EBV



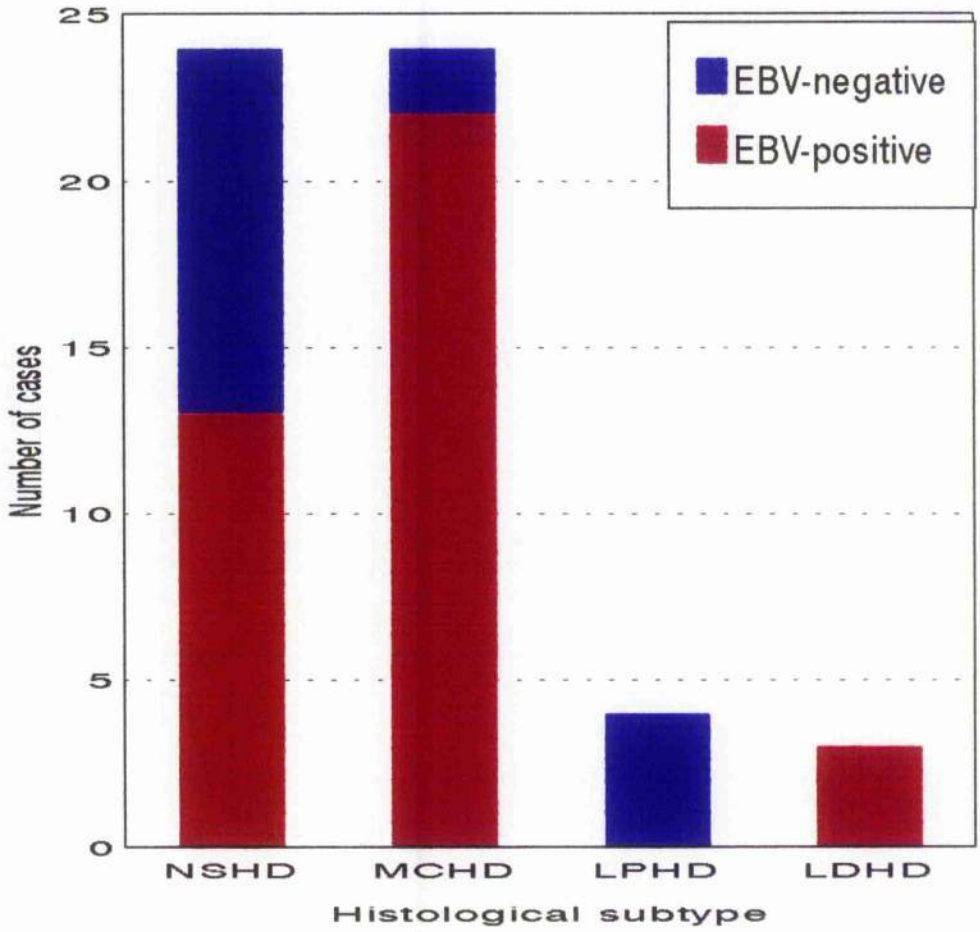
Comparison of EBV-association between paediatric and young adult HD cases from the UK, $p<0.001$

5.3.4 EBV-association by histological subtype and sex

A smaller proportion of NSHD cases were EBV-associated as compared to MCHD and this difference was highly significant (OR=0.11 with 95% confidence limit=0.01-0.63, p=0.008, Figure 5.6). EBV was not detected in the 4 LPHD cases examined, however all 3 of the LDHD cases were EBV-associated. Females were less likely to be EBV-positive than males (11/18 compared to 27/37) however this difference is not statistically significant (OR=0.59 with 95% confidence limits 0.15-2.32).

Figure 5.6 EBV-association by histological subtype

Paediatric HD cases



5.4 Conclusions

In this study, examination of paediatric HD cases from three different geographical locales revealed that EBV was present within the RS cells of affected tissues in the majority of cases. These results substantiate our previous findings in this age group in which 7/13 (54%) of childhood HD cases from the UK were shown to be EBV-associated (Jarrett *et al.*, 1991a).

Until recently, few studies had investigated the association between EBV and paediatric HD (Libetta *et al.*, 1990, Weinreb *et al.*, 1992, Coates *et al.*, 1993, Ambinder *et al.*, 1993, Khan *et al.*, 1993a, Chang *et al.*, 1993a). Analyses from the UK and United States of America (USA) indicated that EBV-positivity rates varied in paediatric HD cases. These results were dependant on the assay used to detect EBV in HD biopsy material (Weinreb *et al.*, 1992, Coates *et al.*, 1993, Ambinder *et al.*, 1993). In a UK series of paediatric HD cases, the expression of EBV LMP-1 protein was detected in RS cells in 37/77 (48%) of cases (Weinreb *et al.*, 1992). Similar levels of EBV-positivity were obtained on investigation of childhood HD cases in the USA (Ambinder *et al.*, 1993). Using a less sensitive assay system, DNA *in situ* hybridisation, Coates *et al.* (1993) detected EBV-positivity in only 5/24 (25%) of cases of paediatric HD. In general, a limited number of cases has been studied; the small number of cases reflects the rare presentation of HD in childhood in Western countries (Mork *et al.*, 1990, McWhirter & Petroschevsky, 1990, Stiller *et al.*, 1991).

Studies of EBV in fixed HD biopsy material from different countries have been possible through the use of recently developed *in situ* techniques. In this study a sensitive RNA *in situ* hybridisation assay was utilised to detect EBV in tumour

tissue. This technology enabled the study of EBV-association in paediatric HD from two additional countries, Brazil and Saudi Arabia.

The proportion of EBV-associated cases was higher in Saudi Arabia and Brazil as compared to the UK although differences were not statistically significant. There is some support for the notion that higher EBV-association rates are related to differences in disease pattern as the odds ratio for EBV-association in Brazil compared to the UK decreased to <1 following adjustment for age and subtype. On comparison with the UK cases, the Brazilian cases were younger (mean age 10.6 years) and the majority were of MCHD subtype. Although in this study Brazil is considered representative of a developing country it is possible that socio-economic development in the Sao Paulo area is leading to a shift in the disease pattern. These observations may explain the lower EBV-association rate in Brazil compared to that reported in other studies from Honduras and Peru (see below).

Consistent results have been reported during the investigation of EBV-positivity in HD cases from Honduras and Peru (Ambinder *et al.*, 1993, Chang *et al.*, 1993a). All of the childhood HD cases from Honduras were EBV-associated. In the same study, EBV was detected in only 9/25 (36%) of paediatric HD cases from the USA (Ambinder *et al.*, 1993). A study of HD in Peru was not designed specifically to investigate paediatric HD, although the age range of the cases was typical of that seen in developing countries (Kirchhoff *et al.*, 1980, Talvalkar, *et al* 1982, Merk *et al.*, 1990, Riyat, 1992). The proportion of paediatric HD cases <15 years of age was 62% and all of these cases were EBV-associated (Chang *et al.*, 1993a).

Further analysis of the present data indicated that the incidence of EBV-association was much greater in cases under 10 years of age. Of the 27 cases examined in this

age category 24 cases were EBV-associated. Thus HD in this group is clearly an EBV-associated disease. Overall, this study provides no evidence that paediatric HD, within each of the 5-year age groups defined above (see section 5.3.3), is more likely to be EBV-associated in developing countries than in developed countries. Fourteen years of age is conventionally taken as the upper limit of the paediatric age group but this need not reflect a biological distinction and there is likely to be an overlap between the childhood and young adult disease patterns around this age. In this study cases aged 13 and 14 years predominated within the 10-14 year age bracket. Given the decline in the proportion of EBV-associated cases in this group these cases may represent the start of the young adult age incidence peak. Indeed, the age of paediatric patients from the UK at clinical onset was older than observed in developing countries (Weinreb *et al.*, 1992, Khan *et al.*, 1993a, Coates *et al.*, 1993). From this data it is important that the age distribution of cases, geographical locale and histology are taken into account when performing international comparisons.

Several studies including largely adult cases have shown an excess of EBV-positive cases within the MCHD subtype as compared to NSHD (Pallesen *et al.*, 1991a, Weiss *et al.*, 1991, Murray *et al.*, 1992a, Delsol *et al.*, 1992) and in three studies this difference has been shown to be statistically significant (Pallesen *et al.*, 1991a, Weiss *et al.*, 1991, Murray *et al.*, 1992a). The results of the present study indicate that this difference is also evident in paediatric populations. Mixed cellularity HD in Honduras, USA and Peru was highly EBV-associated (Chang *et al.*, 1993a, Ambinder *et al.*, 1993). In a study of HD cases from USA, Mexico City and Costa Rica, Gulley *et al.* (1994) also observed that MCHD subtype was strongly predictive of EBV-positivity in these cases. In keeping with other studies this study indicated that all of the LDHD cases were shown to be EBV-associated however, the LPHD

cases were EBV-negative (Boiocchi *et al.*, 1989, Pallesen *et al.*, 1991a, Weiss *et al.*, 1991).

In order to determine whether the polio model was relevant to EBV in HD, the EBV-positivity rate of paediatric HD cases was compared with a young adult HD (15-24 years) group from the UK (see Figure 5.5 and Chapter 6). Only 11/79 cases were EBV-associated in the young adult age group. The proportion of EBV-associated cases in the paediatric and young adults is markedly different ($p<0.001$).

This study enabled the epidemiological features of HD to be analysed with respect to EBV. The significance of the above results are apparent when testing the *polio model*. The finding that EBV-association is significantly less frequent in young adults in a developed country indicates that an analogy between EBV and poliovirus cannot be used to explain the epidemiologic features of HD with respect to EBV. It does remain possible however that HD in young adults is related to an infectious agent for which the *polio model* does hold. The suggestion by MacMahon in 1966 that different age groups may have different aetiologies would be supported by the results of this investigation of paediatric HD and the subsequent comparison with young adult HD from the UK.

There is no definitive evidence that EBV plays a causal role in HD however the available data suggest that it is likely to play a role in the pathogenesis of a proportion of cases. HD occurring in childhood, particularly under the age of 10 years, is predominantly an EBV-associated disease in both developed and developing countries.

Chapter 6

The relationship between EBV-association, age and histological subtype in Hodgkin's disease

6.1 Introduction

An association between EBV and HD has now been established. Clonal EBV genomes have been detected in HD biopsies and the EBV latent genes LMP-1 and EBERs have been localised to the Reed-Sternberg (RS) cells (Weiss *et al.*, 1987a, 1991, Pallesen *et al.*, 1991a, Wu *et al.*, 1990) (as described in Chapters 3 and 4).

On the basis of the epidemiological features of HD, three distinct groups cases have been identified which correspond to the three age groups 0-14 years, 15-34 years and >49 years. MacMahon proposed that the aetiology of HD in these age groups differed and that the disease in young adults was likely to have an infectious aetiology (MacMahon, 1966).

The risk factors associated with the development of HD in young adulthood are suggestive of a lack of early social contact in childhood (Vianna & Polan, 1978). In contrast, the risk of HD in older persons is not related to these epidemiological factors.

Few studies have examined the epidemiological features of HD with respect to EBV and age at clinical onset. Using Southern blot analysis, the relationship between age and EBV-positivity was investigated in a series of 95 HD cases referred to the LRF Virus Centre (Jarrett *et al.*, 1991a). In this preliminary study EBV-positivity rates differed significantly between different age groups. In the paediatric age group (<15 years; 7/13 cases) and the older age cases (>49 years; 27/38 cases) the majority of cases were EBV positive. A low incidence of EBV-positive cases was found in young adult HD (6/36 cases), in particular in NSHD.

In the previous chapter of this thesis, paediatric HD cases from the UK were further investigated for the presence of EBV using an RNA *in situ* hybridisation assay. Thirteen of the twenty-two UK cases were scored as EBV-positive. Examination of paediatric HD cases from the UK and other geographical locales indicated that cases <10 years of age were most likely to be EBV-associated. These data suggest that EBV is likely to be an important agent in paediatric HD.

As described above, epidemiological data suggest that the young adult age group is most likely to have an infectious aetiology (see sections 1.3.4 and 1.5.4). These cases represent the first age incidence peak in developed countries. The NSHD subtype is the most common form of HD in this age group (McKinney *et al.*, 1989, see section 1.3.2.2). From our previous data these cases tend not to be EBV-associated (Jarrett *et al.*, 1991a). From recent reports MCHD cases have also been shown to be more likely to be EBV-positive compared with NSHD cases (Pallesen *et al.*, 1991a).

Following the publication of our first report which highlighted the association between EBV status and age of HD cases, some groups have reported similar findings (I. Lauder, personal communication). However, the majority of studies failed to detect this association (Boiocchi *et al.*, 1989, Coates *et al.*, 1991, 1993, Wright *et al.*, 1991, Uhara *et al.*, 1991, S. Poppema, personal communication, G. Pallesen, personal communication). We therefore decided to test the hypothesis that EBV status was associated with age and histological subtype in HD. In particular, the EBV-positivity rates in the 15-24 year and 50+ year age groups was examined.

The EBER RNA *in situ* hybridisation assay has now been established as the preferred method of detecting EBV in HD tumour tissue (Armstrong *et al.*, 1992b, see Chapter 4). This method is both sensitive and reliable and allows the localisation of EBV to RS cells. Using this methodology, cases of HD referred to the LRF Virus Centre between 1992-1994 were investigated for the presence of EBV. The distribution of EBV in these cases was subsequently analysed with respect to age at clinical diagnosis and histological subtype.

6.2 Materials and Methods

6.2.1 Clinical samples

Lymph node biopsies from HD patients were referred to the LRF Virus Centre from a number of clinical centres. In total 175 HD cases were investigated. The cases were grouped according to age at clinical onset and histological subtype. Seventy-nine of the samples were from non-selected cases with HD, 83 were selected specifically for age and a further 13 cases were selected for both age and histological subtype.

There was age selection in two age groups. First, cases aged >44 years of age were selected in order to augment numbers in the older adult age group. Secondly, 62 cases from a study of young adult HD in the age group 15-24 years were included. Due to selection in this age group, the previously defined 15-34 year old young adult group has been split into two for the analyses.

The cases were classified using the Rye classification system (Lukes *et al.*, 1966) which included 18 LPHD, 48 MCHD, 12 LDHD and 93 NSHD cases. It was not possible to subclassify four of the cases.

6.2.2 Detection of EBV

Paraffin-embedded lymph node biopsies were obtained from all cases. Previous studies have indicated that HD cases which were EBV-positive by *in situ* studies or Southern blot hybridisation for the detection of clonal EBV genomes can now be designated as *EBV-associated* (see Chapter 4).

6.2.2.1 Immunohistochemical analysis

In 62 cases the expression of LMP-1 was examined using a cocktail of monoclonal antibodies (CS1-4) (Rowe *et al.*, 1987) using methodology described in sections 2.3.6 and 3.2.2.

6.2.2.2 EBER RNA *in situ* hybridisation

Lymph node biopsies from all cases were hybridised with a biotinylated oligonucleotide probe, specific for the EBV EBER-1 RNA, using methodology described in section 2.4 and 4.2.3.

6.2.3 Statistical analysis

The statistical analyses were carried out by Dr F.E. Alexander. The EBV status of the samples was analysed with respect to age and histological subtype with or without inclusion of LPHD cases. LPHD cases were analysed independently as this histological subtype is thought to be a distinct clinical entity (Nicholas *et al.*, 1990, Mason *et al.*, 1994, as discussed in section 1.3.3.1). The statistical package EGRET was used throughout the analysis. The data were analysed using logistic regression which permitted adjustment for age and histological subtype.

6.3 Results

Following examination of 175 cases of HD, 44 cases were found to be EBV-associated. Twenty of the 79 (25%) non-selected HD cases were EBV-positive.

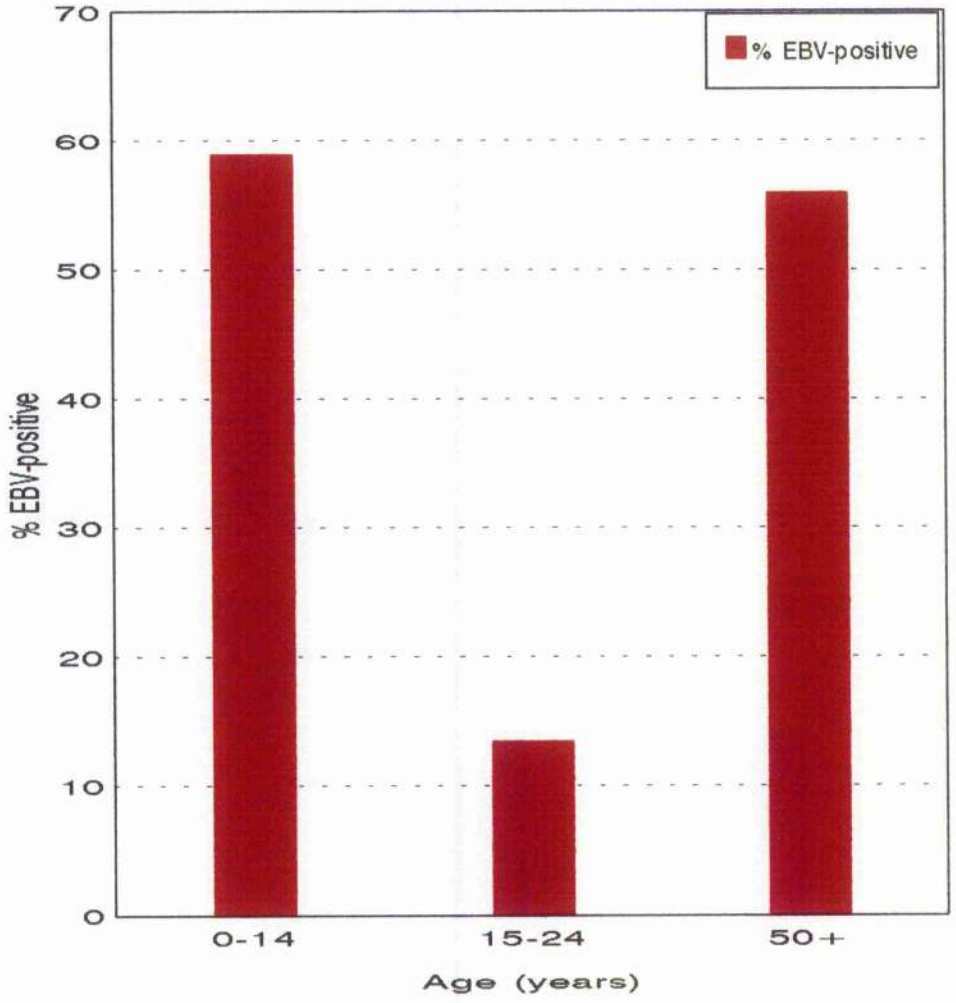
6.3.1 Effect of age

The cases were analysed according to age at date of biopsy, 15-24 years, 25-34 years and 50+ years of age. These particular age divisions were chosen because of the age selection criteria used. The distribution of EBV-associated cases by age, excluding LPHD cases in the 15-24 and 50+ year age groups, is shown in Figure 6.1. Samples which were selected on the basis of both age and histological subtype were excluded from the age analysis. Further analysis of the 35-49 years age group was of limited value as case numbers in this age group were small and cases within the >44 years of age group were highly selected.

When LPHD cases were excluded from the analyses, 11 of the 76 (14.5%) HD cases were EBV-positive within the 15-24 year age group. EBV was detected in 4 of the 14 (28.6%) cases within the 25-34 year age group. In the 50+ year age bracket, 23 of the 41 (56%) HD cases were EBV-associated. Overall, statistical analysis for the effect of age indicated that EBV-positivity increased with increasing age (Table 6.1). The differences in EBV-association by age group are highly significant ($p<0.001$), and statistical significance was maintained when LPHD cases were included in the analyses.

The distribution of EBV-association by histological subtype and age is illustrated in Figure 6.2. Overall, a smaller proportion of NSHD cases (6/40 EBV-positive) were

Figure 6.1 EBV-association by age



Results of EBV-association in paediatric HD cases are described in Chapter 5. LPHD cases were excluded from the analysis of EBV-association in the 15-24 years and 50+ years age.

Figure 6.1 Analysis of EBV-association by age and histological subtypes

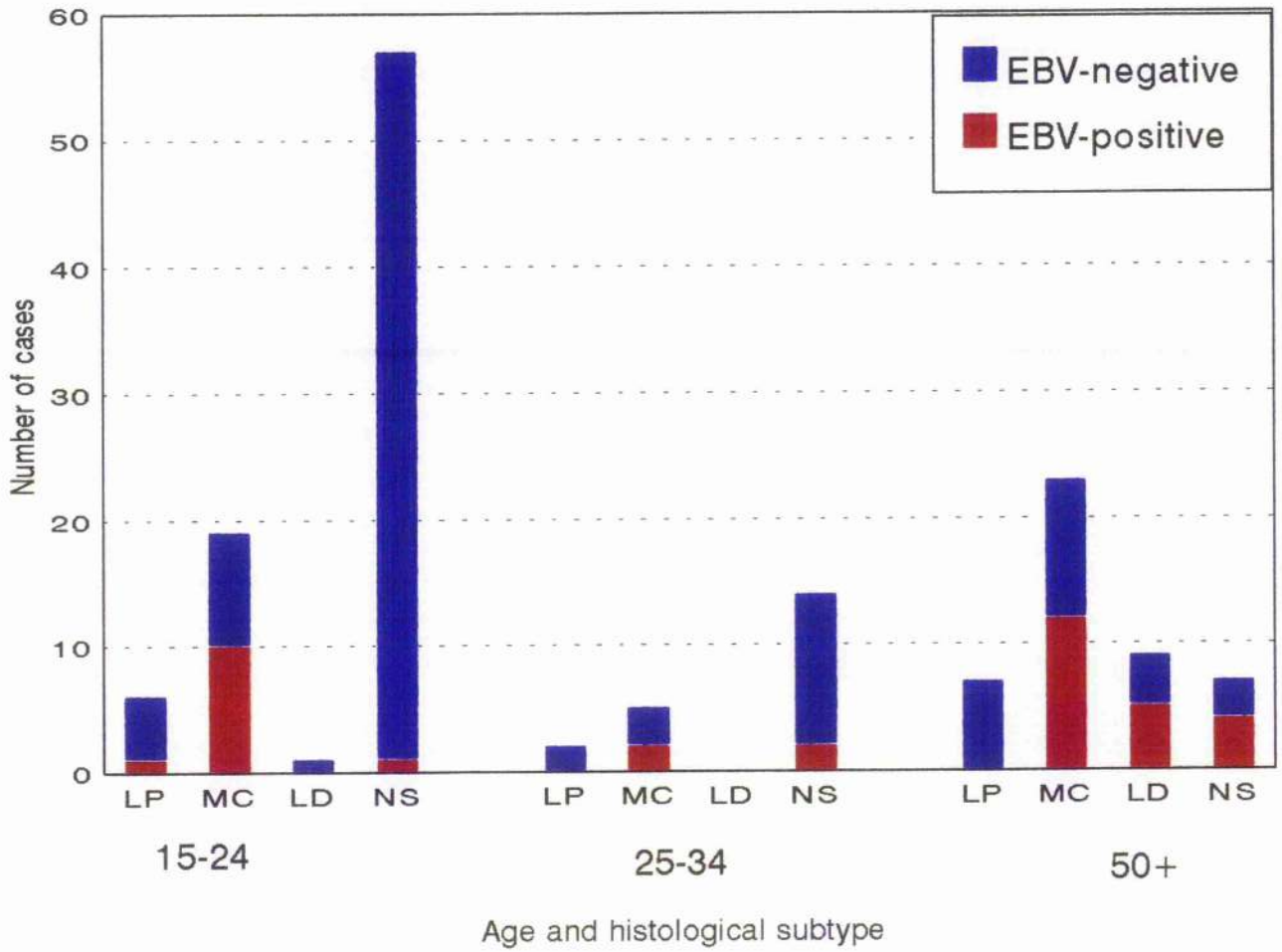
				Age (years)			
	Factors adjusted for	LP	Selection codes used	15-24 OR (95%CL)	25-34 OR (95% CL)	50+ OR (95% CL)	p value
1	None	Y	1-2	1.00[1]	2.09 (0.57-7.66)	5.75 (2.43-13.59)	<0.001
2	None	N	1-2	1.00[1]	2.40 (0.64-9.02)	7.77 (3.16-19-08)	<0.001
3	NS	Y	1-4	1.00[1]	1.74 (0.49-6.20)	2.5 (0.99-6.35)	0.14
4	NS	N	1-4	1.00[1]	1.83 (0.47-7.15)	3.1 (1.10-8.74)	0.055

Selection codes: 1, none; 2, age; 3, subtype; 4, age and subtype

[1] reference category; OR, odds ratio; 95% CL, 95% confidence limits

NS, nodular sclerosis HD; LP, lymphocyte predominance HD

Figure 6.2 EBV-association by age and histological subtype



EBV-associated compared with the MCHD (12/27 EBV-positive). The majority of cases within the young adult age range (15-24 years of age) were NSHD, however only 1/57 of these cases was EBV-positive. Following adjustment for histological subtype, the effect of age is still shown to be statistically significant (Table 6.1).

Comparison of EBV-association between the paediatric HD cases from the UK described in Chapter 5 and the young adult age group (15-24 years of age) showed a highly significant difference ($p<0.001$).

6.4 Conclusions

The results of the present study emphasise the relationship between EBV-association and age in HD. Following exclusion of LPHD cases in the analysis, the majority of older adult cases were shown to be EBV-associated. In contrast, young adult HD cases were less likely to be EBV-positive. These results confirm our previous findings that EBV is associated with a significant proportion of paediatric and older age HD cases, however young adult HD cases are generally non-EBV associated. It has been established from the present study that the relationship between EBV-association and age in HD is non-random.

As histological subtype and age are confounding variables they should not be analysed independently (Jarrett *et al.*, 1991a). Therefore, it is important to record the method of case selection in order to allow accurate statistical examination. There is a general consensus that MCHD cases are more likely to be EBV-positive than NSHD (Weiss et al., 1987a, Boiocchi et al., 1989, Pallesen *et al.*, 1991a, Murray *et al.*, 1992a). In contrast to our previous study (Jarrett *et al.*, 1991a), we have detected EBV-positivity within a significant proportion of MCHD cases compared with NSHD cases. Analysis of the present data indicated that EBV-association by age was significant even when the data were adjusted for the effect of histological subtype. These findings are consistent with the epidemiological features of HD; the risk factors of developing HD are more strongly associated with age than histological subtype.

HD in older cases has been associated with a poor prognosis and survival rate as detailed in section 7.1. In this age group, the detection of EBV increased with increasing age. The majority of cases were EBV-positive aged 50+ years, in

particular cases of MCHD and LDHD subtype. From previous studies, the epidemiological features of older HD cases are different from young adult HD cases and thus are not thought to be associated with a delayed reaction to a common infectious agent. It has been documented that older age cases may have a different host response to EBV. Older persons have been shown to have an overall decreased immune function which may result in an increase in viral load of EBV which in turn may increase the likelihood of developing EBV-associated HD.

As mentioned in section 6.1, the epidemiological features of HD in young adult are suggestive of an infectious aetiology. The majority of young adult cases are NSHD subtype, however these cases are non-EBV-associated. It was within this group of cases that most evidence for the involvement of another infectious agent has been proposed. Examination of the association between other oncogenic DNA viruses and HD is described in Chapter 8.

Few studies have investigated the association between EBV and age (Boiocchi *et al.*, 1989, Libetta *et al.*, 1990, Uhara *et al.*, 1991, Coates *et al.*, 1993) and the majority of reports have failed to detect any association by age in EBV-positive cases. One such study detected EBV in 25 of 77 cases of HD using immunohistochemical and *in situ* hybridisation techniques (Khan *et al.*, 1993a). The EBV-positivity rates between the children, young adult cases and older age cases showed no statistical difference. Although there are differences in the distribution of cases by age and histological subtype between this study and the present study, these features of the cases cannot explain the discrepancies in EBV-association by age between the two studies. Due to the different selection criteria of cases direct comparisons between studies are difficult to interpret.

In the present study, the age distribution of EBV positive cases supports the multiple aetiology hypothesis proposed by MacMahon (1966). The young adult NSHD cases therefore are likely to be a distinct clinical and epidemiological group which should be studied in greater detail in future studies of HD.

Chapter 7

Prognostic significance of EBV-association in Hodgkin's disease

7.1 Introduction

In general, patients with Hodgkin's disease (HD) have a good prognosis with 60% surviving disease-free at 10 years (Proctor *et al.*, 1991). Despite improvements in the treatment of HD, there are still a number of cases with poor clinical outcome. It is necessary to identify these cases in order to initiate alternative treatment regimes early in disease. In order to identify these cases, a prognostic index was devised at the Royal Victoria Infirmary, Newcastle-Upon-Tyne which incorporates haemoglobin, peripheral blood absolute lymphocyte count, clinical stage (Ann Arbor) and age (as a quadratic function) (Proctor *et al.*, 1991). This index will identify those patients of different Ann Arbor stages who have a 75% chance of dying of HD within four years of diagnosis.

As discussed in detail elsewhere in this thesis, EBV is involved in a proportion of cases of HD (Weiss *et al.*, 1987a, Pallesen *et al.*, 1991a, Jarrett *et al.*, 1991a, Delsol *et al.*, 1992). However, EBV is detectable within the RS cells of only 40-50% of cases and these cases have been defined as *EBV-associated* (Armstrong *et al.*, 1992b, see Chapter 4). A number of studies have reported that MCHD cases are more likely to be EBV-associated than NSHD cases (Weiss *et al.*, 1987a, 1989, Staal *et al.*, 1989, Pallesen *et al.*, 1991a, Herbst *et al.*, 1991a, Murray *et al.*, 1992a). In some studies, poor clinical outcome has been associated with older age and also with MCHD histology (Walker *et al.*, 1990). We therefore speculated that EBV-association might be associated with a poorer prognosis.

Using immunohistochemical and *in situ* hybridisation techniques, EBV has been reliably detected within paraffin-embedded biopsy material (Pallesen *et al.*, 1991a, Herbst *et al.*, 1991a, Wu *et al.*, 1990, Weiss *et al.*, 1991, Delsol *et al.*, 1992, Khan *et*

al., 1992). EBV LMP-1 antibodies and probes for the detection of EBV EBERs are now commercially available and can be used in routine testing of HD cases (see Chapters 3 and 4 of this thesis). The present study was devised to assess whether there is a relationship between EBV-positivity and clinical outcome, and also to assess whether EBV positivity could be usefully incorporated into the prognostic index as an additional variable.

7.2 Materials and Methods

7.2.1 Clinical samples

Cases of HD diagnosed over a 13 year period (1976-1989) in the same Pathology laboratory at the Royal Victoria Infirmary, Newcastle-Upon-Tyne (RVI) were identified by Dr Anne Lennard and Dr Brian Angus. Fifty-nine patients were included in the study: 35 males, 24 females. Sixteen patients had Stage I, 13 Stage II, 19 Stage III and 11 Stage IV disease. The prognostic indexes were calculated on prospectively collected data, but do not include additional weighting for bulk disease as this information was not available.

The index has not been validated on seven patients under the age of 15 years. Lymph nodes from all 59 patients were examined by the same pathologist (BA). There were 27 NSHD, 28 MCHD, 3 LPHD and one case of LDHD. In subsequent analyses the cases were classified as NSHD or all other sub-types (AOS).

All cases were treated at the RVI. Early stage disease was treated with radiotherapy alone. Later stage or bulky disease was treated with a four drug regimen (chlorambucil, vinblastine, procarbazine and prednisolone), plus or minus radiotherapy. Minimum follow-up was 48 months.

7.2.2 Detection of EBV

Sections from paraffin-embedded lymph node biopsies were investigated for the presence of EBV using immunohistochemical and *in situ* hybridisation techniques (see sections 3.2.2 and 4.2.3). The results of the EBV analyses of 27 of these

cases were included in a previous report which compared the above techniques (Armstrong *et al.*, 1992b and Chapter 4 of this thesis).

7.2.3 Statistical analysis

The statistical analyses were carried out by Dr F.E. Alexander. The EBV status of the samples was analysed with respect to age, histological subtype, remission, total and disease-free survival and prognostic index. The statistical package EGRET was used throughout. Cases who never achieved remission were classified by EBV status; 2 x 2 tables were analysed with and without age stratification using exact methods. The Cox proportional hazard method was used to compare survival from the date of diagnosis with EBV-association. For analysis of disease-free survival, cases who never attained remission were classified as having failed at time = 1 month. Multivariate analyses permitted adjustment for age and prognostic index. In the tables hazard ratios for EBV-association and 95% confidence limits are provided. A hazard ratio of 1.00 indicates that survival in the two groups is identical whilst a hazard ratio of less than 1.00 corresponds to improved prognosis of EBV-associated disease. Survival curves are based on Kaplen-Meier estimates of survival probabilities.

7.3 Results

In total 21 of the 59 HD cases were found to be EBV-associated. There was almost complete concordance between the results of the 2 assays for EBV; in one case the LMP-1 protein was present in RS cells but EBER-1 was not detected and in another case EBER-1 was detected in the absence of the LMP-1 protein. The cases were categorised into three age groups, paediatric 1-14 years, young adult 15-34 years and older >35 years of age.

Prognosis differed by age and by histological subtype although the latter differences were slight. All the paediatric cases included in this sample achieved full remission and experienced disease-free survival for the duration of follow-up. It follows that these cases contribute no information to any age-adjusted analyses of the effect of EBV status on survival. Within the young adult age group (15-34 years) relapse was more common for the NSHD cases than the remainder of this age group although the differences did not approach statistical significance. Four of the eight relapsers in this group would now be defined as high index, or poor risk patients and would receive more aggressive treatment from diagnosis. The oldest age group had poorest prognosis.

7.3.1 Remission by EBV-status

The EBV status and age group of the patients who achieved remission are shown in Table 7.1. A slightly higher proportion of EBV-associated cases, compared to EBV-negative cases, achieved remission however the number of cases who failed to achieve remission was small and the difference does not approach statistical significance ($p=0.81$). The results are influenced by the paediatric group, all of

Table 7.1 Proportion of cases achieving remission by EBV status and age

	Age (years)			
	0-14	15-34	>35	TOTAL
EBV-positive	4/4	11/11	4/6	19/21
EBV-negative	3/3	22/27	7/8	32/38

which achieved remission, and the young adult group, within which all the EBV-associated cases achieved remission. The number of older adult cases is small but there is no evidence of a higher remission rate in the EBV-associated cases in this age group.

7.3.2 Survival by EBV-status

There is weak evidence of improved disease-free survival and total survival for the EBV-associated cases when no adjustment for age is included in the model (Figures 7.1a and 7.1b and Table 7.2). Following stratification by age (Table 7.2) some differences in total survival remain, although confidence intervals are wide, however differences in disease-free survival are no longer apparent (Figures 7.1a and 7.1b). When the adult age groups are analysed separately there is no consistent pattern with some indication that the EBV-associated cases have improved survival in the young adult group but not in the older group. There were only six HD deaths in the young adults (5 cases of NSHD and one case of AOS) and none of these was EBV-associated.

7.3.3 Prognostic index

Values of the prognostic index were slightly higher (indicating poor prognosis) for the EBV-associated cases. When the data were tested for an independent additional contribution to prognosis, EBV status was statistically significant for total survival but not for disease-free survival. Stratification by age group reduced the magnitude of the contribution and removed its statistical significance.

Figure 7.1a Disease-free survival by EBV status

---------- EBV-positive cases, ———— EBV-negative cases

Figure 7.1b Total survival by EBV status

---------- EBV-positive cases, ———— EBV-negative cases

Figure 7.1a Disease-free survival by EBV status

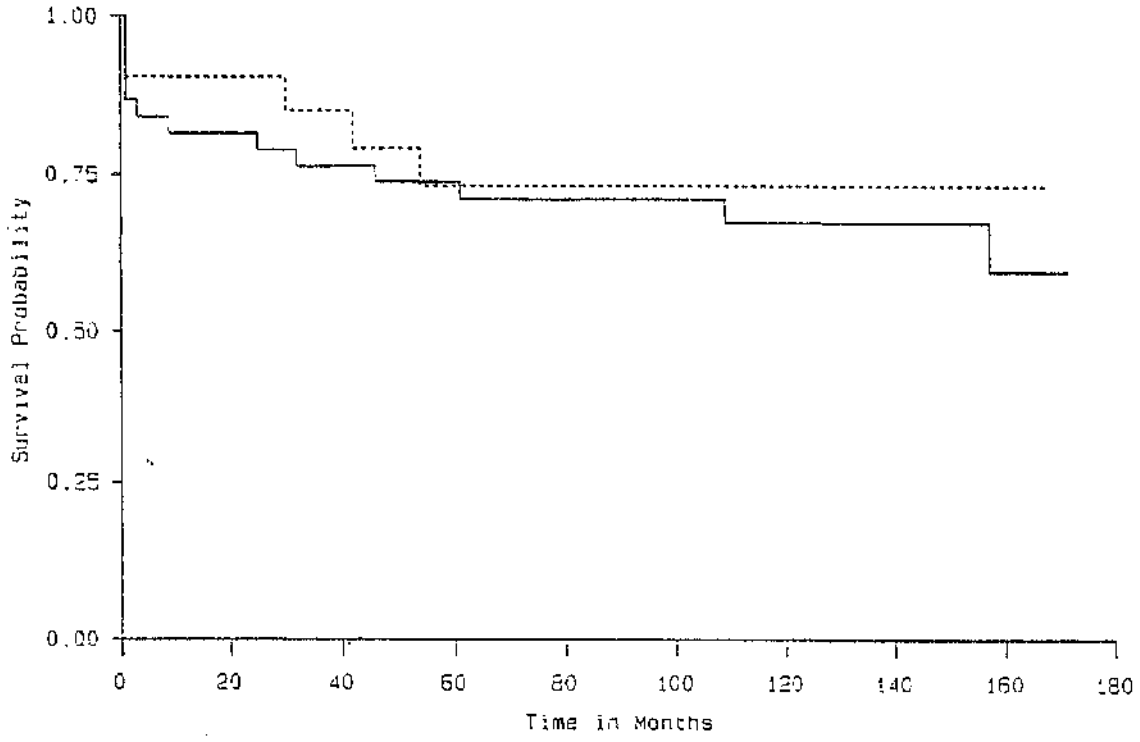


Figure 7.1b Total survival by EBV status

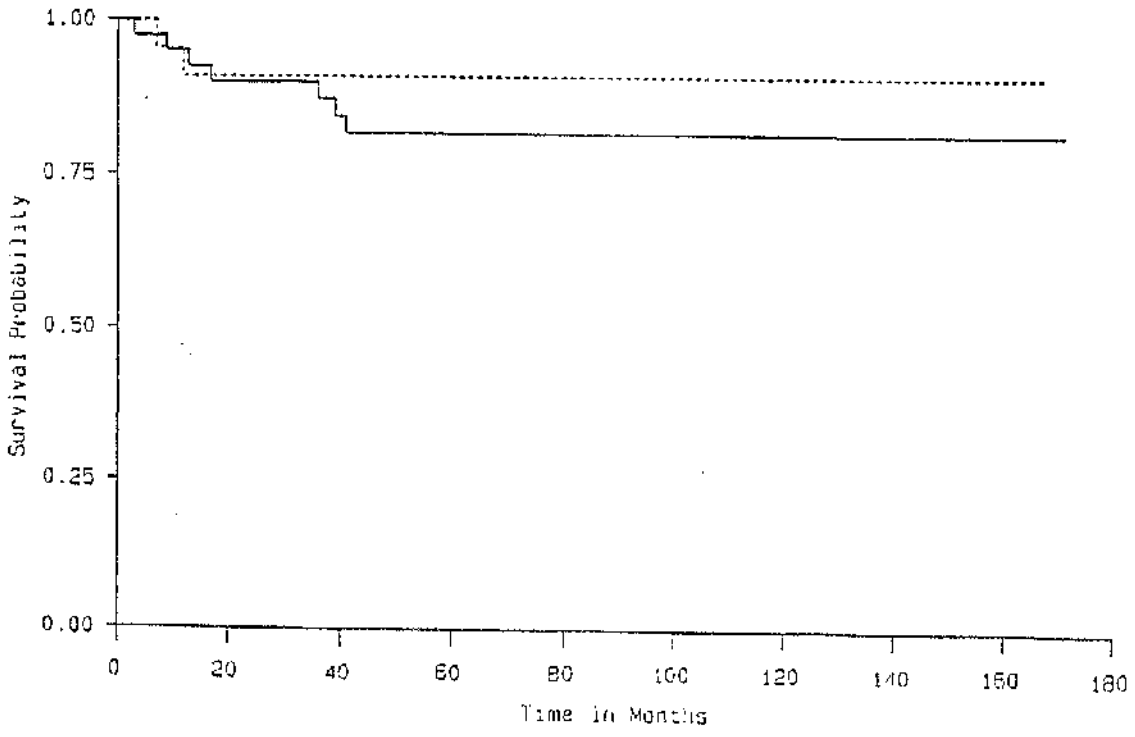


Table 7.2 Disease-free survival of EBV-associated cases

	Stratified by age	Hazard ratio for EBV-positivity	95% Confidence Limit
Disease-free survival	NO	0.76	0.27-2.15
Total survival	NO	0.56	0.11-2.68
Disease-free survival	YES	0.93	0.32-2.65
Total survival	YES	0.56	0.11-2.73

7.4 Conclusions

In this study, we analysed the effect of EBV status on clinical outcome in HD. Of the 59 cases examined, 21 (35%) were categorised as EBV-associated.

There was no evidence of an association between EBV status and survival which was apparent at all ages. Weak associations were observed within specific age groups but were not statistically significant and may be attributed to chance. In the young adult group the EBV-associated cases were more likely to achieve remission and had improved survival. The study provides no evidence to support the hypothesis that EBV-associated cases of HD have a less favourable clinical outcome than EBV-negative cases. It is possible that EBV-status may provide a subtle improvement to the prognostic index. However we feel that these effects are more likely to be attributed to the interplay of chance, small numbers and specific combinations of clinically disparate age groups.

Two previous studies specifically designed to address the association between EBV status and clinical outcome have reached similar conclusions (Vestlev *et al.*, 1992, Fellbaum *et al.*, 1992). Vestlev *et al.* (1992) examined samples from 66 Danish patients using immunohistochemistry and LMP-1 antibodies to detect EBV. Their results indicated that LMP-positivity was not a useful prognostic marker. Fellbaum *et al.* (1992) analysed 130 cases using the polymerase chain reaction (PCR) and reported that EBV positivity did not influence survival time. We have found, however, that PCR is less reliable than *in situ* hybridisation techniques for determining whether cases are EBV-associated (Armstrong *et al.*, 1992b and Chapter 4).

The analysis of the EBV status of HD tumours using immunohistochemistry or *in situ* hybridisation is straightforward and reliable and reagents are readily available. Examination of larger numbers of cases will establish whether EBV-associated cases form a distinct subgroup of HD cases. However, the results of the present study emphasise that the detection of EBV in individual HD cases is not a clinically useful prognostic marker.

Chapter 8

Absence of oncogenic DNA viruses in Hodgkin's disease

8.1 Introduction

It is clear from the studies described in the previous chapters of this thesis that EBV is involved in a proportion of cases of HD. Young adult HD cases are least likely to be EBV-associated (Jarrett *et al.*, 1991a, see Chapter 6). However, it is within this age group that there is most evidence for involvement of an infectious agent in HD.

Investigation of HD cases for an association with infectious agents has concentrated mainly on the antibody response to viral antigens with particular reference to the herpesviruses (Catalano & Goldman, 1972, Henderson *et al.*, 1973, Langenhuysen *et al.*, 1974, Hesse *et al.*, 1977). Raised antibody levels to HSV -1 and -2 (Catalano & Goldman, 1972, Hesse *et al.*, 1977), HCMV (Langenhuysen *et al.*, 1974) and VZV (Henderson *et al.*, 1973) have been observed in HD, although results for HSV-1 and HCMV are not consistent between studies (Levine *et al.*, 1971, Hesse *et al.*, 1977, Evans & Gutensohn, 1984). There is no molecular evidence to support a role for these viruses in the aetiology of HD (Weiss *et al.*, 1987a, Coates *et al.*, 1991, Uhara *et al.*, 1990).

The recently identified HHV-6 and HHV-7 have a worldwide distribution and have been shown to be lymphotropic (Salahuddin *et al.*, 1986, Frenkel *et al.*, 1990). Neither virus has been directly associated with any malignant disease *in vivo*. Recent serological studies have observed elevated antibody titres to HHV-6 in several lymphoproliferative diseases. In a case-control study Clark *et al.* (1990) reported elevated antibody titres to this virus in HD cases and increases relative to controls were most marked in young adults. At present, molecular studies have failed to directly link HHV-6 with HD (Jarrett *et al.*, 1988, Torelli *et al.*, 1991, Gompels *et al.*, 1993). From these results it has been suggested that elevated titres to HHV-6 in HD may be due to reactivation of the virus; alternatively the

virus may be acting as a marker for another infection which is directly involved in HD. HHV-7 is a candidate for the latter role.

In order to eliminate the involvement of known viruses in HD we specifically targeted members of the Adenovirus, Polyomavirus and Herpesvirus families for investigation using a Southern blot assay. These DNA viruses have not been associated with any malignancies in humans. However, the E1A gene of adenovirus, SV40 large T antigen, and polyoma viruses including the LPV have all been shown to have transforming ability *in vitro* (Ruley, 1983, Colby & Shenk, 1982, Gallimore *et al.*, 1974, Takemoto *et al.*, 1982) and have the ability to cause tumours in experimental animals (reviewed by Brady & Salzman, 1986).

Clinical samples from cases of HD, previously studied for the presence of EBV, NHL and reactive conditions were further examined for the presence of adenovirus, SV40, LPV and HHV-7.

8.2 Materials and Methods

8.2.1 Clinical samples

Lymph node samples from 25 cases of Hodgkin's disease were investigated for the presence of the E1A gene of adenovirus, SV40, LPV and HHV-7. Cases were classified according to the Rye classification system (Lukes *et al.*, 1966) and included 15 cases of NSHD, 7 cases of MCHD, one case of LDHD and one case of LPHD.

Twenty three samples of NHL were also included in the study. These cases included low and high grade lymphomas and three cases of angioimmunoblastic lymphadenopathy (AILD). In addition 18 reactive conditions were examined which included 12 reactive lymph nodes from paediatric patients, two cases of systemic lupus erythematosus (SLE), two cases of Sjögren's syndrome and two cases of lymphadenopathy of unknown cause.

DNA was extracted from all samples using the procedures described in section 2.2.1.

The HHV-7 molecular analyses were carried out on samples included in this survey. Experiments were carried out by Dr Ruth Jarrett and Alice Gallagher in collaboration with Dr Z. Berneman, Hematologie, Univ. Ziekenhuis Antwerpen, Edegem, Belgium.

8.2.2 Analysis of high molecular weight DNA

The restriction endonuclease digestion of DNA, separation of DNA fragments and Southern blot hybridisation procedures are described in detail in section 2.6.

Briefly, ten μg of DNA were digested with the restriction enzyme Hind III or Pst I (Life Technologies Inc., Paisley, UK) and incubated overnight at 37°C. The restriction endonuclease digested high molecular weight DNA was separated on a 0.8% agarose gel in TBE buffer (section 2.6.2, Appendix I). Placental DNA was included as a negative control. Positive controls included plasmid DNA which was digested and diluted to a level equivalent to a single copy gene present in a 10μg sample of genomic DNA.

The high molecular weight DNA was transferred to nylon membrane (Hybond N, Amersham International plc, Little Chalfont, UK) using a Southern blot procedure (section 2.6.3).

8.2.3 Preparation of DNA probes

Using standard procedures, plasmid DNA was prepared as described in sections 2.5.1-2.5.3.

8.2.3.1 Adenovirus E1A probe

The pLA1 construct contains the Bgl IID fragment of adenovirus type 5 from nucleotide 1 to 3322, cloned between the EcoR I and BamH I sites of pAT153. The plasmid pLE1A, supplied by Dr V Mautner, Institute of Virology, University of Glasgow is truncated at nucleotide 1704 and contains the entire E1A gene but no E1B coding sequences (Dery *et al.*, 1987). The plasmid DNA was digested with the restriction enzyme Hinc II (Life Technologies Inc., Paisley, UK) which yielded an E1A region probe of 1.7 kb. The plasmid DNA fragment was gel purified as described in section 2.5.3.1.

8.2.3.2 Lymphotropic papovavirus (LPV)

The biologically active molecular clone of LPV strain K38 (pL6) was kindly provided by Dr M. Pawlita, Institit fur Virusforschung, Hiedelberg, Germany. The plasmid DNA was digested with the restriction enzyme BamH I (Life Technologies Inc., Paisley, UK) which generated a complete LPV probe of 5.1 kb. The plasmid DNA fragment was gel purified as described in section 2.5.3.1.

8.2.3.3 SV40

Supercoiled circular SV40 DNA derived from strain 776, propagated in BSC-1 cells, was supplied by Life Technologies Inc. This DNA was linearised with BamH I (Life Technologies Inc., Paisley, UK).

8.2.3.4 pBR322

Following digestion with the restriction enzyme Pst I (Life Technologies Inc., Paisley, UK), pBR322 was used as probe in order to determine whether contaminating plasmid sequences were present in DNA samples.

8.2.3.5 TCRβ gene probe

In order to assess the integrity of the DNA under analysis, the TCRβ region probe, C91β, a cDNA clone of the β chain obtained from the HTLV-1 infected cell line C91PL (Gledhill *et al.*, 1990) was utilised in these investigations. To obtain a probe for the TCRβ constant region the plasmid was digested with EcoR I and Xho I (both Life Technologies Inc., Paisley, UK) to generate a constant region probe of 900bp. The plasmid DNA fragment was gel purified as described in section 2.5.3.

8.2.4 Radioactive labelling of DNA

This procedure has been summarised in section 2.5.4. Plasmid inserts from the E1A gene of adenovirus, LPV and TCRβ, linearised pBR322 and SV40 were radio-labelled with α^{32}P dCTP using a random priming procedure (Multiprime labelling kit, Amersham International plc, Little Chalfont, UK).

8.2.5 Molecular analysis

The nylon filters were hybridised with the radio-labelled probes (see section 2.5.4) under conditions calculated by the formula:

$$T_m=81.5 + 16.6\log M + 0.41(\%G+C)-500/n-0.61(F)$$

M is the ionic strength in moles/L, G+C is the percentage of G and C, n is the length of probe and F is the percentage formamide in the hybridisation solution. For every 1% mismatching of bases in a DNA duplex the T_m is reduced by 1°C

The T_m calculatod for pLE1A, pL6, pBR322 and TCRβ probes was approximately 92°C. The hybridisation kinetics have been shown to be optimal at approximately 25°C below the T_m for dsDNA probes, thus the filters were hybridised at 65°C in 3x SSC (Appendix I) in the absence of formamide. The filters were washed as detailed in section 2.6.4 and placed in a cassette containing an intensifying screen.

8.3 Results

The detection of viruses in HD, NHL and reactive conditions is summarised in Table 8.1. Previous results indicated that EBV was detected in Reed-Sternberg (RS) cells in 14 of these cases of HD.

In order to test the integrity of the sample DNA the TCRβ probe was hybridised to the filters. Only cases which had clearly visible bands on autoradiographic film were retained in the study.

Initial screening of DNA samples for the presence of adenovirus type 5 E1A gene identified four cases which gave a signal on the autoradiographic film after one to seven days exposure. The four cases detailed above were further investigated using the restriction enzyme Pst I (Life Technologies Inc., Paisley, UK) and hybridised with the pBR322 probe. The bands identified on the original blots were shown to be due to plasmid sequences contaminating the DNA samples.

Subsequent analysis using the LPV and SV40 probes failed to detect these viruses in any of the clinical samples tested.

From a total of 50 cases of HD which included all 25 cases analysed in this study, there was no evidence of HHV-7 sequences in any clinical samples (R.Jarrett and Z. Berneman, personal communication).

Table 8.1 Detection of DNA viruses in HD, NHL and reactive conditions

		Number of cases positive				
Cases	Number tested	EBV*	Adenovirus	LPV	SV40	HHV-7
HD	25	14	0	0	0	0
NHL	23	NT	0	0	0	0
Reactive	18	0	0	0	0	0

HD, Hodgkin's disease; NHL, non-Hodgkin lymphoma; reactive, reactive conditions

NT, not tested; *, EBV detected in RS cells

EBV, Epstein-Barr virus; LPV, lymphotropic papovavirus; SV40, simian virus 40; HHV-7, Human herpesvirus-7

8.4 Conclusions

It has been suggested that another infectious agent may be involved in the pathogenesis of young adult HD. This study was designed to examine the presence of the herpesvirus, HHV-7, adenovirus and polyomaviruses in HD. The experimental method chosen was Southern blot hybridisation which has been previously been shown to allow detection of a single genome if present in ~2% of cells of a DNA sample derived from 1 x 10^6 cells. In HD, the putative malignant cell population of RS cells are present in only 1-5% of the total cellular infiltrate. The sensitivity of the Southern blot assay is thus on the borderline for detection of virus in RS cells. The Southern blot assay therefore is suitable for use as an initial screening technique. The increased sensitivity associated with PCR methodology would overcome the problems associated with detection of virus in HD samples. However, the use of this assay to screen samples in this first instance may fail to detect defective or related viruses. The ideal method of detecting virus in HD requires sensitivity and the ability to demonstrate the cellular localisation of the virus.

This study examined the presence of adenovirus and polyomaviruses in human malignant disease at a molecular level. These viruses were specifically chosen due to their ability to transform mammalian cells (see sections 1.4.4, 1.4.5 and 1.4.6)

The EIA and EIB region of adenovirus codes for the transformation function of the virus (Sambrook *et al.*, 1974). The E1A sequence of adenovirus is well conserved over the different subtypes of adenovirus. Despite the oncogenic potential of these viruses in cell culture and animal models there was no evidence for adenovirus sequences in any of the HD, NHL and reactive

conditions investigated in the present study. Further studies are now underway to investigate these cases using the E1A gene from adenovirus type 12.

During the search for potential infectious agents which may be important in the aetiology of HD, we studied members of the papovavirus family: LPV and SV40. It has previously been reported that human sera have antibodies that react with LPV of African green monkeys (Brade *et al.*, 1980, Takemoto *et al.*, 1982), however no human equivalent virus has been isolated. In order to eliminate these viruses from our investigations for a novel viral agent we studied both HD and NHL cases. To date we have not detected these viruses in any clinical sample.

Other candidate viruses include members of the herpesvirus family. The most thoroughly examined human herpesvirus is EBV and this virus has been associated with a proportion of cases of HD and NHL. As stated previously, EBV was detected in a proportion of these cases of HD.

Two herpesviruses, HHV-6 and 7, have recently been isolated and they have been shown to be distinct from other herpesviruses at the molecular level. The initial isolation of HHV-6 from six patients with various lymphoproliferative diseases (Salahuddin *et al.*, 1986) suggested that there may be an association between HHV-6 and lymphoproliferative disease. The oncogenic potential of HHV-6 was reported by Razzaque (1990). HHV-6 has the ability to transform NIH 3T3 cells and these transformed cells cause tumours in nude mice. This oncogenic potential of HHV-6 is in keeping with the majority of herpesviruses (reviewed by MacNab, 1987), although the viral oncogenes in HSV-1, HSV-2 and CMV have not been identified (Cameron *et al.*, 1985, Galloway *et al.*, 1984).

HHV-6 DNA sequences have been detected in tissue samples from patients with malignant disease (Jarrett *et al.*, 1988, Josephs *et al.*, 1988). However, HHV-6 sequences were not detected in any of the HD or NHL cases in this series using Southern blot analysis (R. Jarrett, personal communication). In contrast to these findings, using a PCR technique, Torelli *et al.* (1991) detected HHV-6 DNA in 3 of 65 HD cases, however HHV-6 was absent in the 41 NHL cases studied (G. Torelli, personal communication). HHV-6 DNA was observed in only two of these cases using a Southern blot analysis. It has been shown previously that the sensitivity of PCR enables amplification of HHV-6 sequences which are present in infected lymphocytes and therefore a direct role for HHV-6 has still to be clarified. The detection of HHV-6 within RS cells in HD using *in situ* hybridisation would clarify the role of HHV-6 in lymphoproliferative disease. One such study detected HHV-6 positive cells in lymphocytes, however the virus was not present in RS cells (Krueger *et al.*, 1989).

HHV-7 was isolated by Frenkel *et al.* (1990), from stimulated, purified CD4+ T cells from a healthy individual. Further isolates from chronic fatigue syndrome and saliva from healthy persons (Berneman *et al.*, 1992, D. Clark, personal communication) have been reported. There does appear to be a widespread distribution of the virus and infection occurs early in childhood. Serological studies have shown elevated titres to HHV-7 in non EBV-associated cases of HD, however these differences did not reach statistical significance (R.Jarrett, personal communication). In this study, in collaboration with Dr Z. Berneman, there was no evidence of HHV-7 infection in cases of HD or NHL.

In conclusion the lack of known potentially oncogenic DNA viruses in HD, NHL and reactive conditions has strengthened the evidence that an as yet unidentified virus may be involved in the non EBV-associated HD cases. Studies to identify

novel viral sequences from single RS cells are now ongoing in the LRF Virus centre.

Chapter 9

General Discussion

It is only recently that molecular evidence linking EBV with HD has been reported. As discussed in section 1.5.4, EBV genomes have been detected in a proportion of HD biopsies using Southern blot hybridisation. Despite the presence of clonal EBV genomes in HD cases, it has been recognised that the detection of viral nucleic acid in samples from affected tissues is not sufficient to establish a relationship between a virus and disease (section 1.6).

HD is an unusual malignancy in which the tumour cells, RS cells, constitute only a minority of the tumour mass (section 1.3.2). Thus, the detection of EBV in RS cells would strongly support an aetiological role for EBV in HD. In view of this, the initial investigations of this project were designed to determine the cellular localisation of EBV within HD biopsy material (Chapters 3 and 4).

The demonstration of the EBV latent gene product, LMP-1 exclusively in RS cells in HD has indicated that EBV is localised to the malignant cells in HD. The transcription and translation of a latent gene, with oncogenic potential, in RS cells supports the idea that EBV is likely to be involved in the disease process in HD (Chapter 3). Further support for this concept was presented by the expression of LMP-1 in the majority of HD cases previously shown to have clonal EBV genomes.

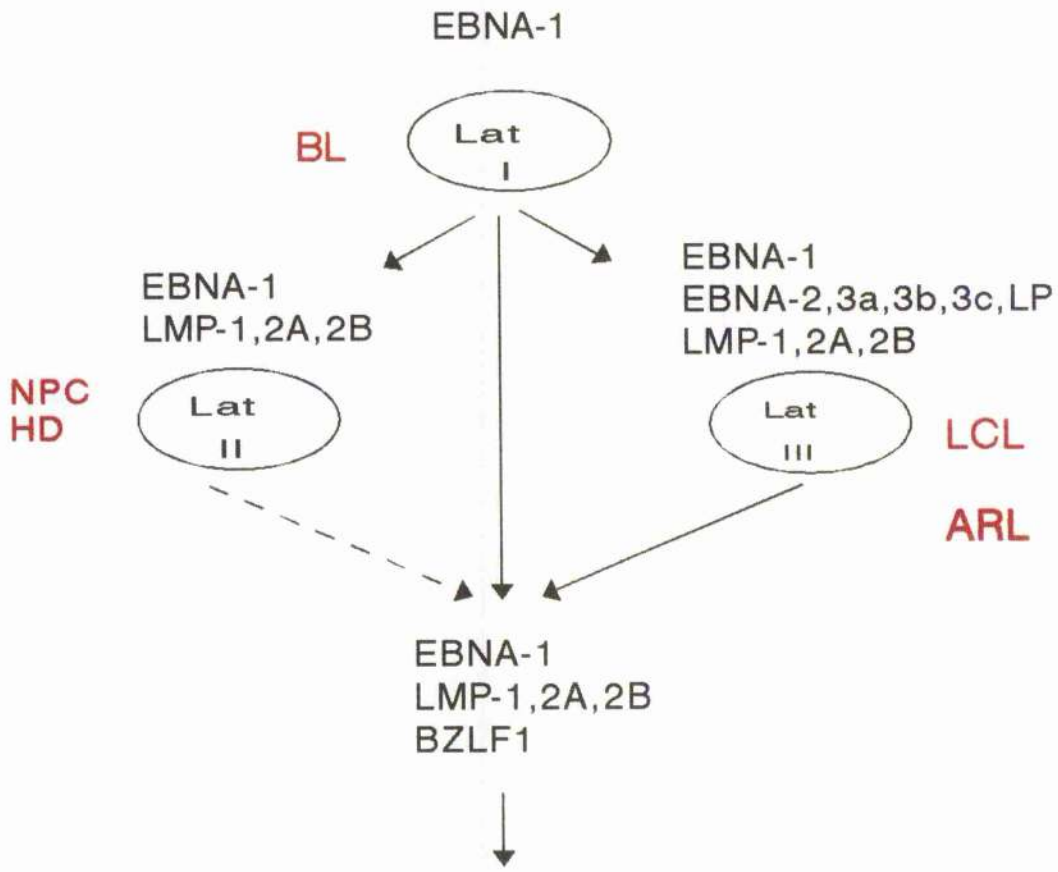
Using immunohistochemical staining expression of LMP-1 was detected in RS cells, in the absence of EBNA-2 expression. Due to a lack of effective antibodies for the other EBV latent genes, Deacon *et al.* (1993) examined the pattern of EBV latent gene expression in EBV-positive HD at the transcriptional level. A distinct and consistent pattern of viral transcription was identified which included the expression of EBNA-1, LMP-1, LMP-2A and 2B. As EBNA-1 is required for the maintenance of the viral genome in episomal form, it was assumed that the EBNA-1 protein was

expressed in HD however until recently there was no direct evidence for this. EBNA-1 expression in RS cells has now been confirmed in a proportion of HD cases using a monoclonal antibody (P. Murray, personal communication). Overall, these results indicate that the pattern of EBV latent gene expression in HD is similar to that seen in the epithelial malignancy, NPC. This pattern of latency is distinct from other patterns of EBV latency observed in EBV-associated malignancies, *in vivo* (see section 1.4.5). The latency pattern associated with HD, consisting of EBNA-1, LMP-1 and LMP-2, has been described as Lat II (Figure 9.1). The BZLF-1 protein, an early lytic cycle protein, has only been detected within the nuclei of a minority of RS cells within a minority of HD cases (Pallesen *et al.*, 1991b, Brousset *et al.*, 1993a). Thus in the majority of cases of HD, EBV infection in RS cells is tightly latent. In general, the pattern of EBV latent gene expression is less heterogeneous in HD than in other EBV-associated malignancies.

In order to further elucidate the role of LMP-1 in HD, the expression of LMP-1 was correlated with the presence of various cellular genes. From previous work on B-cells *in vitro*, LMP-1 has been shown to have the ability to induce a number of cellular genes including CD23, CD40 and bcl-2. It is clear from the results presented in Chapter 3 that CD23 is rarely detected in RS cells. Furthermore, there is no correlation between the detection of CD23 and LMP-1 expression in RS cells. As discussed in Chapter 3, the absence of EBNA-2 expression in HD may a significant factor in the lack of upregulation of CD23.

Expression of the LMP-1 protein has been shown to induce the expression of bcl-2 and this in turn is thought to contribute to the development of tumours by the inhibition of apoptosis thereby increasing cell survival. Although the t(14;18) translocation which is involved in the dysregulation of bcl-2 has been detected in a

Figure 9.1 Patterns of EBV latent gene expression



BL, Burkitt's lymphoma; NPC, nasopharyngeal carcinoma; HD, Hodgkin's disease; LCL, lymphoblastoid cell lines (lymphomas in immunosuppressed persons);ARL, AIDS-related lymphoma

small number of HD cases, bcl-2 expression has not been consistently detected in RS cells (Chapter 3). It would appear that the expression of bcl-2 as a result of induction by LMP-1 or the t(14;18) translocation was not consistently detected in HD.

CD40, a member of the nerve growth family receptor family, has also been investigated in HD. CD40 has been shown to mediate several effects on B-cells *in vitro* including prevention of apoptosis in germinal centres (Liu *et al.*, 1989) which is accompanied by the expression of bcl-2. Despite high levels of CD40 expression in RS cells, the expression of CD40 was not shown to correlate with either bcl-2 or LMP-1 expression (O'Grady *et al.*, 1994). Although these studies have not clarified the role of LMP-1 in HD, the results suggest that LMP-1 acts on cellular genes in HD in a manner distinct from that described in B-cells *in vitro*.

One specific feature of LMP-1 which requires further investigation is the finding that LMP-1 protein is highly immunogenic and has the ability to generate EBV-specific cytotoxic T lymphocyte (CTL) reponses in healthy individuals (Murray *et al.*, 1988, 1992b). The expression of LMP-1 in HD would imply that there is an immune defect in HD, a concept suggested in previous studies (Kumar & Penny, 1982). The possibility that the downregulation of the immunodominant EBNA-3 family of proteins in HD may help RS cells evade EBV-specific CTL surveillance has been proposed. However since LMP-1 and LMP-2, which are both immunogenic, are both present in HD, it is unclear how RS cells evade immune-destruction. One possibility may be through variations in LMP-1 sequence which may represent less immunogenic forms of EBV (Knecht *et al.*, 1993).

The LMP-2 peptide epitope presented by HLA A2.1 has recently been identified (Lee *et al.*, 1993). As LMP-2 is present in RS cells, studies have been designed to determine the prevalence of HLA A2 in HD. It has been suggested that EBV-positive HD may be more prevalent among HLA A2-negative persons. Poppema & Visser (1994a) recently reported that the expression of HLA A2 in HD did not correlate with EBV status.

The suggestion that a defect in HLA class I expression in HD may account for the lack of a CTL response has also been investigated. In a recent study, strong staining for HLA class II in RS cells was evident, however HLA class I expression was absent in most cases (Poppema & Visser, 1994b). These results require further investigation as this may be an important mechanism through which RS cells escape immune recognition.

Comparison of techniques to detect EBV in HD material indicated that the RNA *in situ* hybridisation is the most useful, sensitive and reliable method of determining EBV latent infection. From the results of the studies described in Chapter 4, HD cases which were EBV-positive either by *in situ* studies or Southern blot hybridisation for the detection of clonal EBV genomes were designated *EBV-associated.* As the EBER RNA is present consistently in all RS cells of EBV-positive HD cases, this assay is ideal for the investigation of the epidemiological aspects of HD in relation to EBV.

The heterogeneous nature of HD has been highlighted by the detection of EBV in specific groups of cases. EBV was associated with paediatric HD cases <10 years of age, older adult cases and MCHD cases (Chapters 5 and 6, Armstrong *et al.*, 1993). Paediatric HD cases from different geographical locales were strongly EBV-

associated which leads us to believe that EBV is important in the pathogenesis of HD in these cases. A poorer prognosis has been associated with older age and MCHD cases. However, in the present studies described in Chapter 7, EBV was not shown to be a useful prognostic marker in HD (Armstrong *et al.*, 1994).

The identification of EBV within specific subgroups of HD suggests that this disease may comprise of a range of conditions which have various aetiologies, some of which are strongly associated with EBV. This situation is similar to that observed in BL where eBL is strongly associated with EBV however in a proportion of cases of BL the virus is not detected in the tumour cells. In BL the presence of the myc translocation, as described in section 1.5.1, is detected in both EBV-positive and EBV-negative BL cases. It is likely that EBV is one step in a multi-step process leading to development of a tumour.

Although the epidemiological features of HD suggest that young adult HD is most likely to be caused by an infectious agent (section 1.3.4), this group of cases is less frequently associated with EBV (see Chapter 6). We speculate that another as yet unidentified infectious agent may be involved in these cases.

The above observation prompted the studies described in Chapter 8. Viruses with known oncogenic potential were specifically chosen for investigation. The failure to detect these viruses in HD samples may be because they are present at low copy number. Therefore an assay which incoporates the extreme sensitivity required to detect a single copy viral gene within a tumour cell population of HD is essential. An assay which allows the amplification of low copy viral DNA combined with the added advantage of the localisation of the virus to malignant cells would be particularly useful in situations, such as HHV-6, where the virus has been detected

previously in the reactive infiltrate of HD. One such assay has recently been described, PCR *in situ* hybridisation (Hasse *et al.*, 1991).

From the above data, there is no absolute proof that EBV is directly involved in the pathogenesis of HD. There is a growing list of diseases in which EBV has been detected including Ki-1 anaplastic large cell lymphoma (Herbst *et al.*, 1991b, Carbone *et al.*, 1993), gastric carcinoma with lymphoid stroma (Oda *et al.*, 1993), lethal midline granuloma (Harabuchi *et al.*, 1990, Borisch *et al.*, 1993), pyrothorax-associated pleural lymphoma (Fukayama *et al.*, 1993) and enteropathy-associated T-cell lymphoma (Pan *et al.*, 1993). EBV has not been detected in lymphomatoid papulosis (Kadin et al., 1993), hairy cell leukaemia or monocytoid B-cell lymphoma (Chang *et al.*, 1993b) although an infectious aetiology is suspected for these conditions.

Due to the scarcity of RS cells in HD tumour tissue and a lack of a distinct immunophenotype for these cells, there are problems associated with the study of HD material. Few cell lines have been derived from HD biopsy material and those which have been successfully cultured tend to have originated from late stage HD material (V. Diehl, personal communication). To date, no animal model for HD has been established. Recently, the severe combined immunedeficient (SCID) mouse, transplanted with HD biopsy material, was examined as a potential model system for HD. All of the mice produced tumours which had a B-cell phenotype and resembled EBV-positive human B-cell lymphoproliferations (Kapp *et al.*, 1993, A. Krajewski, personal communication). It was thought that this animal model would allow investigation of RS cells independent from a host response, however these EBV-positive tumours are more likely to have been derived from EBV-infected small bystander lymphocytes present within the cellular infiltrate. These studies indicate

that the SCID mouse model for HD is not an adequate system to determine the histogenesis of RS cells.

The above observations indicate that it is particularly difficult to propagate RS cells in culture therefore it would appear that another method of examination of HD biopsy material is required. Analysis at the single cell level, where the RS cells are investigated independently of the cellular infiltrate, has been recently reported. Trumper *et al.* (1993) described a single-cell based PCR technique in which cDNA sequences from single RS cells were analysed. This technique showed that the expression pattern of lineage markers in RS cells was heterogeneous between cases and that single RS cells coexpressed genes characteristic of several haematopoietic lineages. In addition, mutation in exon 7 of the p53 gene was present in five out of seven single RS cells from the one case studied. It has previously been shown using immunohistochemical methods that p53 is overexpressed in HD. These results highlight the possibility that mutant p53 may be involved in the neoplastic process in a proportion of HD cases. There was no correlation of p53 with the EBV status of HD lesions (Niedobitek *et al.*, 1993).

Further application of the single cell PCR method has allowed the investigation of the origin of the malignant cells in HD. Using a single cell PCR method, Roth *et al.* (1994) examined the presence of IgH gene rearrangements in RS cells. This group failed to detect Ig gene rearrangements in single RS cells. However using a similar strategy two groups recently reported rearrangements of Ig genes in single RS cells (H.Stein and ML. Hansmann, personal communication to R. Jarrett). These techniques may allow the identification of an interaction between epidemiological, environmental and genetic events in HD.

The application of the single cell PCR assay, as mentioned above, to HD tumour biopsy material would assist in the identification of novel viral sequences at the RNA level in single RS cells specifically in non EBV-associated cases. These studies are now underway at the LRF virus centre.

Appendices

Appendix I

Buffers and Solutions

TNE buffer for purification of high molecular weight DNA	Sodium chloride Tris pH 8 EDTA adjusted to pH 8	144mM 10mM 1mM
TE buffer	Tris pH 8 EDTA pH 8	10mM 1mM
TAE buffer	Tris base Sodium acetate Sodium chloride EDTA adjusted to pH 8	40mM 20mM 20mM 2mM
TBE buffer	Tris base Boric acid EDTA adjusted to pH 8	90mM 90mM 2.25mM
Alkali buffer	Sodium hydroxide Sodium chloride adjusted to >pH 12	0.5M 1.5M
Neutralising buffer	Tris base Sodium chloride Conc. HCl adjusted to pH 8	0.5M 3M 3.3%
Elution buffer for removal of DNA fragments from DEAE membrane	Sodium chloride Tris pH 9 EDTA	1M 50mM 1mM
SSC	Sodium chloride Sodium citrate	0.15M 0.015M
Denhardt's solution (100x)	Ficoll Bovine serum albumin Polyvinyl Pyloridine (PVP)	2% 2% 2%
Southern hybridisation buffer	Formamide Denhardt's solution Sodium dodecyl sulphate Tris pH 7.4 EDTA pH 8 SSC Dextran sulphate Genebloc	As appropriate 5x 0.1% 50mM 10mM 3x 10% 250μg/mL

Appendix II

Solutions for Immunohistochemical staining I

TBS	Sodium chloride	0.15M
	Tris pH 7.6	0.05M
Lugol's Iodine solution	Iodine	40mM
	Potassium iodide	120mM
Scotts Tap Water substitute (STWS)	Potassium bicarbonate (w/v)	0.2%
	Magnesium sulphate (w/v)	2%
Fast red substrate	Naphthol AS-BI phosphate	40mg/200mL
	Dimethyl formamide (v/v)	0.5%
	Tris pH 8.2	0.1M
	Levamisole	5mM
	add Fast red TR salt	1mg/mL
NBT/BCIP substrate	Tris pH 9	0.1M
	Sodium chloride	0.1M
	Magnesium chloride	5mM
	add NBT	0.33mg/mL
	add BCIP	0.16mg/mL

Appendix II cont'd

Solutions for Immunohistochemical staining II

Stock solution of NBT	NBT	75mg/mL
	Dimethyl formamide	75%
Stock solution of BCIP	BCIP	50mg/mL
	Dimethyl formamide	100%
Mayer's haematoxylin	Haematoxylin in IL distilled water	0.1%
	Potassium or aluminium sulphate	5%
	Citric acid	0.1%
	Chloral hydrate	5%
	Sodium iodate	0.02%

Appendix III

Solutions for *in situ* hybridisation

Pronase buffer	Tris pH 7.5	50mM
	EDTA pH 8	5mM
Glycine buffer	Tris pH 7.5	0.1M
	Sodium chloride	0.1M
	Glycine	2mg/mL
Prehybridisation solution	Sodium phosphate	20mM
	Denhardt's solution	1x
	Dextran sulphate	10%
Hybridisation buffer	Formamide	As appropriate
	Dextran sulphate	10%
	Sodium phosphate pH 7.4	20mM
	SSC	3x
	Denhardt's solution	1x
	Genebloc	100µg/mL
	yeast tRNA	125µg/mL
Antibody buffer	BSA	0.1%
	Tris pH 7.5	0.1M
	Sodium chloride	0.1M
	Magnesium chloride	5mM
	Brij 35 (w/v)	0.25%
Wash solution 1	Tris pH 7.5	0.1M
	Sodium chloride	0.1M
	Triton-X 100	0.01%
	Magnesium chloride	5mM
Wash solution 2	Tris pH 9	0.1M
	Sodium chloride	0.1M
	Magnesium chloride	5mM

Appendix IV

Solutions for growth and alkaline lysis of bacteria

Luria-Bertani (LB) medium	Sodium chloride Bacto-tryptone Bacto-yeast extract adjusted to pH 7	170mM 1% 0.5%
SOC medium	Bacto-tryptone Bacto-yeast extract Sodium chloride Potassium chloride Magnesium cloride Glucose adjusted to pH 7	2% 0.5% 10mM 2.5mM 10mM 20mM
L-agar	LB medium with Bacto-agar added	1.5%
STE buffer	Sodium chloride Tris pH 8 EDTA adjusted to pH 8	0.1M 10mM 1mM
Solution 1	Tris pH 8 EDTA pH 8 glucose	25mM 10mM 50mM
Solution 2	Sodium hydroxide SDS	0.2M 1%
Solution 3	Potassium acetate (5M) glacial acetic acid deionised water	60mls 11mls 28.5mls
Lysozyme solution	lysozyme in H_20	5mg/mL
PCI9	phenol chloroform isoamyl alcohol	50% 48% 2%

Appendix V

Coating glass slides using 3-aminopropyltriethoxysilane (APES)

Prior to coating with the APES solution, the glass slides were soaked in 10% Decon at room temperature overnight, rinsed under tap water for at least one hour and dried overnight at 65°C. The slides were immersed in a 2% solution of APES in acetone for five seconds, rinsed in acetone, washed in distilled water and dried at 37°C overnight. The slides were wrapped in foil and stored at room temperature.

Preparation of diethyl pyrocarbonate(DEPC)-treatment of water

Deionised water was treated with 0.1% diethyl pyrocarbonate (DEPC) and autoclaved for 20 minutes at 15 lb/square inch on liquid cycle.

Preparation of pronase

A stock solution of pronase at 20mg/mL was pretreated at 37°C for two hours to eliminate the DNase present. The solution was aliquoted and stored at -20°C.

Appendix VI

Mixing table for Tris buffers

The stated quantities are for 500mL of a 1M solution which when diluted to 50mM at room temperature will be at the stated pH.

Tris Buffers	pH7.5	pH8.0	pH8.2	pH9.0
Tris HCl	63.5g	44.4g	35.4g	7.6g
Tris base	11.8g	26.5g	33.4g	54.7g

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