Host-virus interactions in Hodgkin's disease

by

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DECLARATION

The work detailed in this thesis is my own original work except where otherwise stated, and has not been submitted for any other degree.

SUMMARY

The pathogenesis of Hodgkin's disease (HD) still poses many questions and this disease is the subject of intense and varied research. There is now little doubt that Epstein-Barr virus (EBV) plays a role in the development of a number of cases, over one third of all HD. The role played by the virus in these cases, however, is as yet not fully understood.

The immunopathology of HD is complex. There is a large reactive infiltrate of immune cells, including lymphocytes, histiocytes and eosinophils, with the tumour cells comprising only around 1% of the total tumour mass. However, despite these infiltrating lymphocytes, among them CD8+ CTLs, the Reed-Sternberg (RS) cells are not eliminated, persisting in the lymph nodes and eventually increasing in number if the patient is left untreated.

In 1992, at the start of the work of this thesis, little was known about the mechanisms by which RS cells evade immune detection. This phenomenon is of particular interest in EBV-associated HD, where the RS cells are known to harbour latent virus and express several viral latent antigens including EBNA-1 and the latent membrane proteins (LMPs). The LMPs are known to be targets for EBV-specific CTLs, therefore why do such CTLs not recognise or eliminate RS cells expressing these proteins?

The answer to this question may fall into one of two broad categories. First, there may be a defect in EBV-specific CTL function in people who develop EBV-associated HD; secondly, there may be a defect in foreign antigen presentation by RS cells that allows these cells to persist and proliferate. The first possibility is investigated in the work presented in chapter 3 of this thesis. EBV-specific CTL responses in the peripheral blood of HD patients were assayed using an *in vitro* technique. EBV-associated HD cases were compared to other HD cases and to healthy subjects. Although the study was small, the findings suggest that there may be a diminished CTL response in EBV-associated HD which is not seen in the other cases and controls.

The second possibility is addressed in chapter 4 of this thesis. It was postulated that people possessing HLA class I antigens known to present epitopes of the LMPs would not develop EBV-associated HD. HLA-A2 was the focus of this study as an epitope of LMP-2 is restricted through this HLA antigen. The results refuted this hypothesis.

The possibility that EBV itself may be the cause of the recognition defect rather than the existence of a presentation defect was also investigated. The sequence of a target LMP-2 epitope presented by HLA-A2 was investigated in selected cases and analysed for the presence of mutations which would abolish recognition of the epitope by CTLs. This hypothesis was also refuted by these studies. In conclusion, there must be other mechanisms by which the RS cells evade immune detection.

ABBREVIATIONS

aa	amino acid
ABC	avidin-biotin complex
AP	alkaline phosphatase
APAAP	alkaline phosphatase anti-alkaline phosphatase
APES	3-aminopropyltriethoxysilane
β_2m	β_2-microglobulin
BL	Burkitt's lymphoma
BSA	bovine serum albumin
CBMC	cord blood mononuclear cells
CsA	cyclosporin A
CTL	cytotoxic T lymphocyte
DMF	dimethyl formamide
DMSO	dimethyl sulphoxide
DTT	dithiothreitol
EA	early antigen
EBER	EBV-encoded RNA
EBNA	Epstein-Barr virus nuclear antigen
EBV	Epstein-Barr virus
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid
EHV	equine herpesvirus
EMA	epithelial membrane antigen
ER	endoplasmic reticulum
FBS	foetal bovine serum
FITC	fluorescein isothiocyanate
HBSS	Hank's balanced salts solution
HCMV	human cytomegalovirus
HD	Hodgkin's disease
HDLD	lymphocyte depleted HD
HDLP	lymphocyte predominance HD
HDMC	mixed cellularity HD
HDNS	nodular sclerosis HD
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid
HHV	human herpesvirus
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
HMW	high molecular weight
HSV	herpes simplex virus
HTLV-1	human T-cell leukaemia virus type-1
Ig	immunoglobulin
IM	infectious mononucleosis
IPTG	isopropyl-β-D-thio-galactopyranoside

IVR	*in vitro* regression assay
KSHV	Kaposi's sarcoma-associated herpesvirus
LCL	lymphoblastoid cell line
LMP	latent membrane protein
LPD	B-lymphoproliferative disease
MHC	major histocompatibility complex
MOPS	3-N-morpholinopropanesulphonic acid
NHL	non-Hodgkin's lymphoma
NIB	non-ionic detergent buffer
NPC	nasopharyngeal carcinoma
OD	optical density
OHL	oral hairy leukoplakia
ORF	open reading frame
PAB	phosphate azide buffer
PAGE	polyacrylamide gel electrophoresis
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PCR	polymerase chain reaction
Rb	retinoblastoma protein
rpm	revolutions per minute
RPMI	Roswell Park Memorial Institute
RS	Reed-Sternberg
SDS	sodium dodecyl-sulphate
TAP	transporter-associated for antigen processing
TBS	Tris buffered saline
TCR	T-cell receptor
TEMED	N,N,N',N'-tetramethylethyldiamine
TPA	12,0-tetradecanoyl phorbol-13-acetate
UV	ultra violet
VCA	viral capsid antigen
VZV	Varicella Zoster virus
X-gal	5-bromo 4-chloro s-indoyl β-D-galactopyranoside

Chapter 1

Introduction

1.1 Introduction to study

Viruses have been shown to play a direct role in the aetiology of malignant disease in a wide range of animal species. In humans it is estimated that around 20% of all cancers are associated with infection by a specific viral agent (Waterhouse *et al.*, 1982; Parkin *et al.*, 1992). In some cases there is strong evidence that the virus plays a causative role in the development of disease. One example of this is the involvement of human T-cell leukaemia virus type-1 (HTLV-1) in adult T-cell leukaemia, for which a large body of scientific evidence exists (Hinuma *et al.*, 1981). For other associations it has been harder to establish that the virus implicated plays a causal role in the development of malignancy.

Epidemiological data suggest that Hodgkin's disease (HD) is likely to have an infectious aetiology. In the last decade an association has been made between certain cases of HD and the lymphotropic herpesvirus Epstein-Barr virus (EBV). EBV has been shown to be present in the tumour cells of HD in a proportion of cases and current evidence suggests that the virus may play a direct role in the pathogenesis of these cases.

EBV is a ubiquitous virus which is carried throughout life by the infected host. The studies described in this thesis aimed to investigate the host-virus equilibrium in HD, particularly in EBV-associated HD. It was postulated that an impaired immune response to EBV could predispose to the development of HD.

In order to investigate this hypothesis the cellular immune response to EBV was measured in HD patients using an *in vitro* system. The study also aimed to determine whether a sub-group of the population may be predisposed to the disease, either by possession of a specific HLA type, or by harbouring a viral strain carrying a mutation.

1.2 Hodgkin's Disease

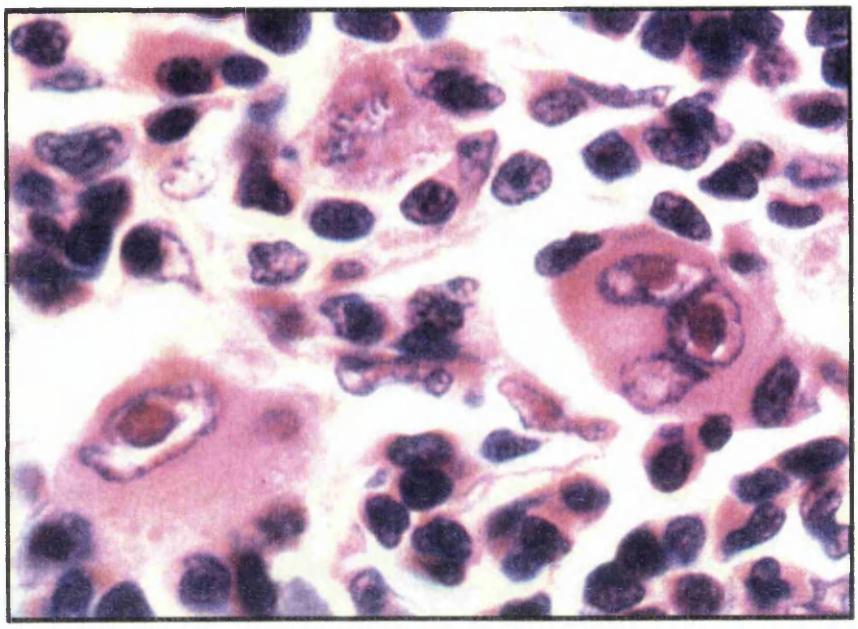
HD is a lymphoma which was first described by Thomas Hodgkin in 1832 (Hodgkin, 1832). At the turn of the century, Reed and Sternberg separately described the large bi- or multi-nucleated cells, which are the main histological feature of the disease (Sternberg, 1898; Reed, 1902); these cells are now referred to as Reed-Sternberg cells (Fig.1.1a). The progressive nature of untreated HD, with spreading lymphadenopathy followed by frequent involvement of the spleen, liver and bone marrow, leading ultimately to the death of the patient, led to the belief that the disease was malignant. By the 1980s this was a well established school of thought (Kaplan, 1980; 1981; Rosenberg, 1989). The demonstration of clonality and frequent aneuploidy in the RS cell population using molecular-based methods supported the theory that HD was indeed a malignancy (Haber *et al.*, 1992; Trumper *et al.*, 1993; Kuppers *et al.*, 1994). A recent report, although controversial, has cast doubt on this subject with the finding that 50% of HD cases studied had polyclonal RS cell populations (Hummel *et al.*, 1995). Further investigation is required to resolve this issue which has important implications for the pathogenesis of HD.

1.2.1 Histopathology of Hodgkin's disease

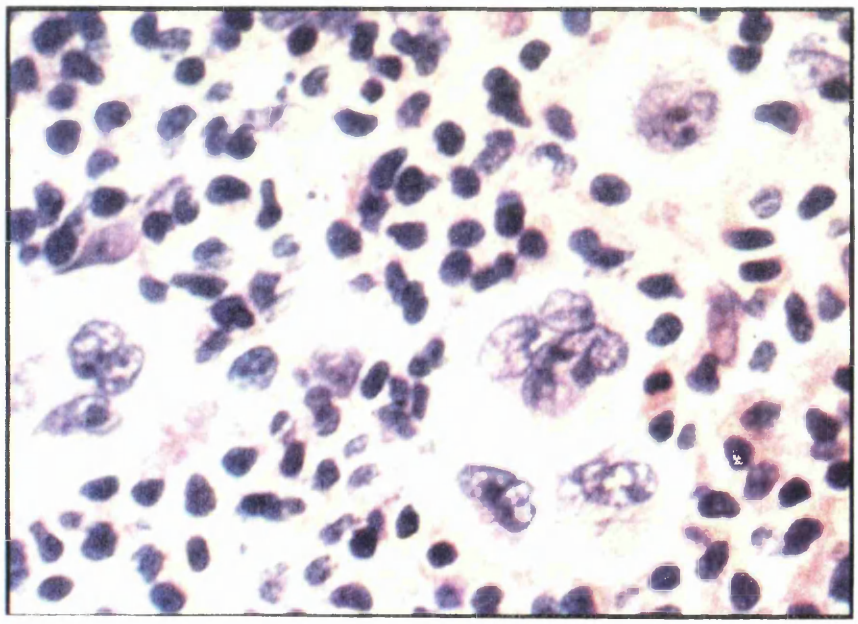
HD has a distinct histological appearance characterised by the presence of RS cells, the putative malignant cells (Fig.1.1a). These are large bi- or multi-nucleate cells with prominent nucleoli. Cells with a similar morphology are seen in other lymphomas and in infectious mononucleosis (Strum *et al.*, 1970; Tindle *et al.*, 1972; Khan *et al.*, 1993b); in order to make the diagnosis of HD the RS cells must be present in an appropriate cellular background (Lukes, 1971). This background consists of T-cells, B-cells, plasma cells, eosinophils and histiocytes. Fig.1.1 shows (a) histological appearance of classical HD with multinucleate RS cells and (b) lymphocyte predominance HD showing "popcorn" cells. The disease is unusual among malignancies in that the RS cells usually make up less than 1% of the total tumour mass, the remainder being the large reactive cellular component.

Fig.1.1 Histological appearance of HD

a. "classical" HD



b. HDLP



(high power)

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Table I.i Rye classification for Hodgkin's disease

subtype	*% of cases*	*criteria for definition*
HDMC	20-40%	obvious RS cells infiltrate mainly small lymphocytes, plasma cells, granulocytes
HDNS	50-70%	RS variant "lacunar" cells and RS cells nodule formation due to subdivision of lymph node by collagen bands
HDLD	<5%	cellular depletion and disordered fibrosis numerous obvious RS cells
HDLP	5-10%	few classical RS cells "popcorn" RS cell variants wide range of other features

HD is divided into four histological subtypes based on the histology - mixed cellularity (HDMC), nodular sclerosis (HDNS), lymphocyte depleted (HDLD) and lymphocyte predominant (HDLP) (Lukes *et al.*, 1966). This nomenclature is known as the Rye classification system and is a simplified version of the original histopathological definition made by Lukes and Butler (1966). Although this classification has remained essentially unchanged for 30 years it is still regarded as clinically and pathologically relevant (Bennett *et al.*, 1991). The Rye classification is outlined in Table I.i.

Recent data suggest that HDLP is a discrete condition which should be classed separately from other forms of HD (Nicholas *et al.*, 1990; Mason *et al.*, 1994). The malignant cells of HDLP are thought to be of B-cell origin and express B-lineage surface markers (Mason *et al.*, 1994). In the recently proposed REAL system for the classification of lymphomas, HD is now divided into two main entities, classical HD and HDLP (Harris *et al.*, 1994).

1.2.2 Nature of the Reed-Sternberg cell

The lineage of the RS cell has been under debate since its first description in the literature in 1898 (Sternberg, 1898). There are currently three candidate lineages; macrophage/monocyte and their myeloid precursors (Hsu *et al.*, 1985), interdigitating or follicular dendritic cells (Delsol *et al.*, 1993; Soderstrom *et al.*, 1994), and lymphoid cells (Stein *et al.*, 1985; Hummel *et al.*, 1995). Characterisation of the RS cell has proved difficult due to the small number of these cells within the total tumour mass. The question of the lineage of the RS cell has been addressed using immunophenotyping and more recently genotyping; results of immunophenotyping studies of frozen biopsy tissue and paraffin-embedded material have been reviewed by Drexler (Drexler, 1992).

RS cells do not consistently express any lineage markers. In a proportion of cases they have been shown to express B-cell markers including CD19, CD20, CD22 and CD79 (Schmid *et al.*, 1991; Knapp *et al.*, 1994). This has led certain groups to suggest that the RS cell is of B-cell origin. However there are also reports of the presence of T-cell markers such as CD2, CD3 and CD4 being expressed (Agnarsson & Kadin, 1989). The markers most consistently expressed by classical RS cells are activation antigens; the most reliable marker is generally accepted to be CD30 (Durkop *et al.*, 1992), an activation marker which is also expressed by lymphoblastoid cell lines (Andreesen *et al.*, 1984) and activated T-cells (Stein *et al.*, 1985). In addition they generally express CD15 (Paietta, 1992), CD25, the interleukin-2 receptor, CD71, the transferrin receptor (Hugh & Poppema, 1992), CD40 (O'Grady *et al.*, 1994) and HLA class II. Adhesion molecules including ICAM-1 (CD54) and LFA-3 (CD58) are also frequently expressed. In contrast to this expression pattern, the characteristic "popcorn" cells of HDLP usually express CD20 and CD45 but are seldom CD15 or CD30 positive (Stein *et al.*, 1991). These cells also express epithelial membrane antigen (EMA) whereas classical RS cells do not.

HD biopsy material, and more recently micromanipulated single RS cells, have been investigated for the presence of immunoglobulin (Ig) and T-cell receptor (TCR) gene rearrangements. Ig gene rearrangements have been detected in DNA extracted from biopsy material in most series investigated; in non-selected series rearrangements have been detected in up to 25% of cases, with more rearrangements demonstrable following enrichment of RS cells or selection of cases with higher RS cell numbers (Weiss *et al.*, 1986;

Brinker *et al.*, 1987; Sundeen *et al.*, 1987; Kuppers *et al.*, 1994). Results of TCR rearrangement studies have been more variable, with only two studies reporting rearrangements in a significant number of cases (Griesser *et al.*, 1987; Herbst *et al.*, 1989). Using micromanipulation and single cell PCR, Ig gene rearrangements have been detected in putative RS cells (Kuppers *et al.*, 1994). As mentioned above, such studies have led to controversy about the clonal nature of the RS cell.

Overall, these data suggest that RS cells are almost certainly of haematopoietic origin and current opinion favours the lymphoid lineage.

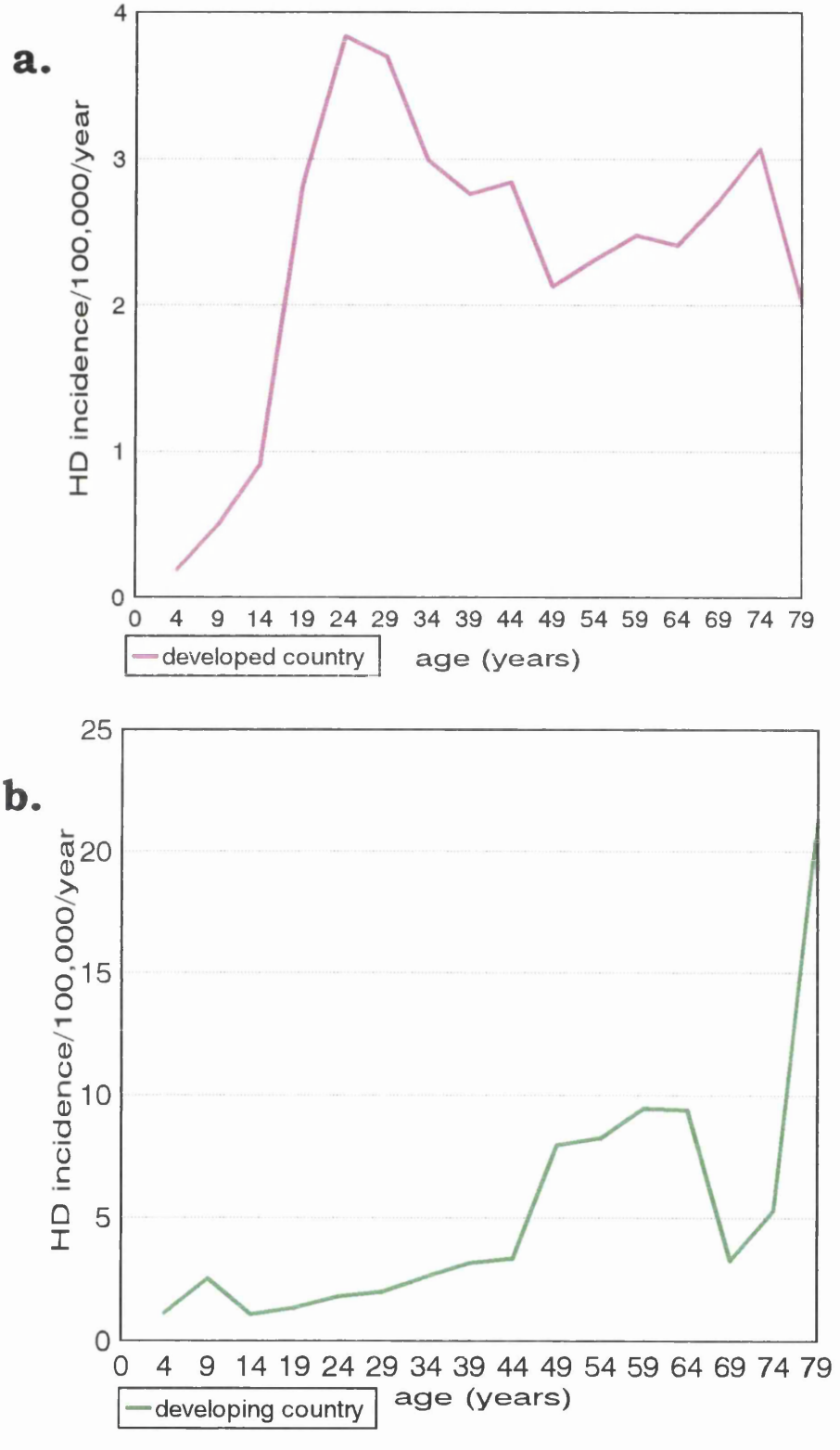
1.2.3 Epidemiology of Hodgkin's disease

HD is not a particularly common cancer overall but one which predominantly affects young adults, being the second most common cancer in this age group. The incidence is $3.0/10^5$ person years in the UK (McKinney *et al.*, 1989) and $2.4/10^5$ person years in the USA (Glaser, 1987).

The age-incidence for HD has a bimodal distribution (Fig.1.2). In developed countries (Fig.1.2a) there is low incidence in early childhood rising to a prominent peak in the young adult (15-34 years) age group, and a second, less pronounced peak in older age (over 50 years). In developing countries the first peak occurs in childhood with low incidence in the young adult age group and a second peak in older adults (Fig.1.2b). An intermediate distribution has been described in countries undergoing socio-economic development and in rural areas of developed countries (MacMahon, 1966; MacFarlane *et al.*, 1995). These differences in the age-incidence curves for different geographical locales led to the suggestion that HD has several epidemiological patterns (Correa & O'Conor, 1971).

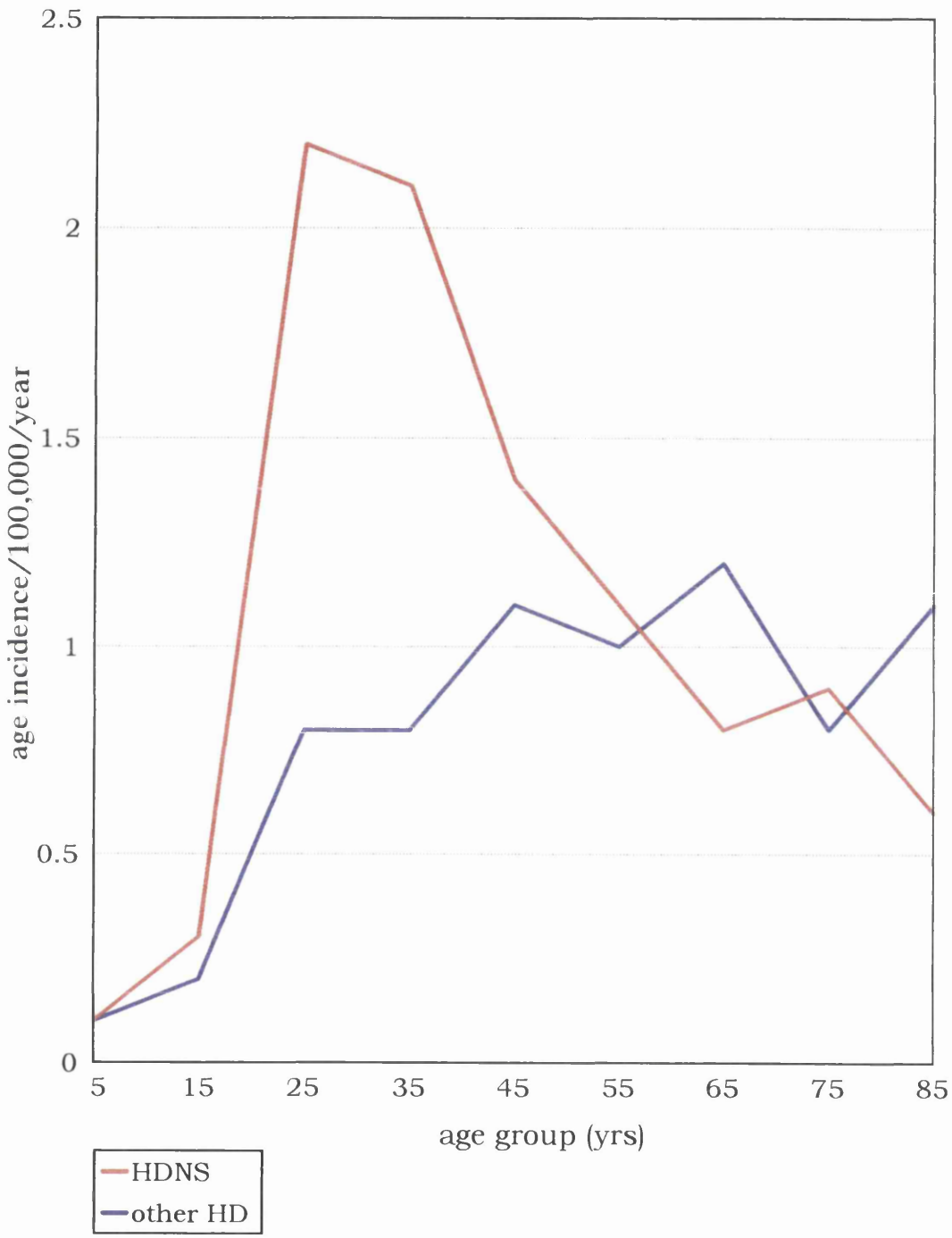
The risk factors for the development of HD in different age groups are distinct. There is consistent evidence suggesting that high socio-economic status is a risk factor in the development of young adult HD (Gutensohn & Cole, 1981; Glaser, 1987; Alexander *et al.*, 1991a). In contrast, the older adult peak and the childhood peak seen in developing countries are associated with lower standards of living (Gutensohn, 1982; Alexander *et al.*, 1991b).

Fig.1.2 Age incidence of HD



Reproduced from data compiled by Correa & O'Conor (1971) and Cartwright *et al.* (1990)

Fig.1.3 Age incidence of HDNS compared to all other HD cases



Reproduced from data compiled by McKinney *et al.* (1989)

The different histological subtypes of HD have different age distributions (McKinney *et al.*, 1989; Glaser & Swartz, 1990; Alexander *et al.*, 1991b). HDNS has a unimodal age-incidence curve and these cases account for the young adult peak seen in developed countries. The other subtypes show a gradual increase in incidence with increasing age and largely account for the second peak (Fig.1.3). There is a male predominance which is most striking in childhood cases and older adults; in the young adult peak the male:female ratio is decreasing (McKinney *et al.*, 1989; Glaser & Swartz, 1990). A recent study investigating international time-trends has shown an increase in young adult and a decrease in older adult HD cases (Hartge *et al.*, 1994).

1.2.3.1 Hypotheses for the aetiology of Hodgkin's disease

In 1966 MacMahon put forward the hypothesis that HD was a heterogeneous condition encompassing at least three disease entities with distinct aetiologies. This was based on the epidemiological features of the three age groups, the histological subtypes and also on prognosis/survival (MacMahon, 1966). He defined three age groups, 0-14 years, 15-34 years and 50+ years and proposed that the aetiologies in these three groups were different. He suggested that HD in the 15-34 year age group involves an infectious agent. Since then a body of data has accumulated suggesting that the risk factors for the young adult and older age group are indeed different. The older age group cases are more similar to non-Hodgkin's lymphoma in this respect. These findings have given rise to the widely accepted "two disease" hypothesis (Gutensohn, 1982; Alexander *et al.*, 1991a).

A second, though not mutually exclusive, hypothesis suggests that HD occurs as a result of delayed exposure to a common infectious agent which would normally be encountered in early childhood (Gutensohn & Cole, 1977). The hypothesis is based on data which found an increased risk of developing young adult HD in persons with high socio-economic status (section 1.2.3). It is thought that such persons lacked early social contact and thus were not exposed to certain childhood pathogens until later in life. A study performed by Paffenbarger *et al.* (1977) showed that college students who developed HD had suffered fewer common childhood illnesses

relative to controls. The hypothesis is referred to as the delayed exposure hypothesis or late host response model. An extension of this theory draws a comparison between the aetiologies of HD and paralytic poliomyelitis in the pre-vaccine era (Gutensohn & Cole, 1981). This was suggested following the finding that there is an inverse relationship between childhood incidence in developing countries and young adult incidence in developed countries (Correa & O'Conor, 1971). This is known as the polio model and is outlined below.

In the pre-vaccine era, living conditions determined the age of infection with polio virus. Infection in childhood was usually associated with mild symptoms and paralysis was rare; infection in adolescence and beyond was much more likely to result in more severe symptoms including paralysis. In developing countries living conditions were poor and thus infection in childhood was usual and paralytic cases were observed in this age group. In developed countries the standard of living was higher and infection was delayed until early adulthood; the result was a peak of paralytic cases in this age group. The similarity was therefore drawn between paralytic polio and HD, and it was hypothesised that the childhood peak seen in developing countries and the young adult peak present in developed countries involved the same infectious agent.

In 1995 MacFarlane performed a new analysis of world HD data sets, including the same data as that analysed by Correa & O'Conor in 1971, and failed to find the same inverse association between incidence rates of HD in childhood and young adults in developed and developing countries. This was largely the evidence on which the polio model was based, therefore the findings in the recent analysis provide further support for the distinct aetiologies hypothesis (MacFarlane *et al.*, 1995).

With regard to the putative infectious aetiology of HD, cluster analysis was carried out in the 1970s and early 1980s, but with inconclusive results (Kryscio *et al.*, 1973; Greenberg *et al.*, 1983), possibly confounded by the long latent period from initial infection to onset of disease. Data from more recent studies suggest weak clustering of HD cases which is consistent with an infectious aetiology involving a virus with a long latent phase, or delayed exposure to a common pathogen (Alexander, 1995).

1.2.4 Aetiology of Hodgkin's disease

Isolation of RS cells from sample material is thwarted by the small proportion of these cells within the overall tumour mass. Attempts at *in vitro* propagation of these cells, to enable further investigation, have been largely unsuccessful; only 15 continuous HD cell lines have been recorded to date (Kapp *et al.*, 1995; Kanzler *et al.*, 1996; Wolf *et al.*, 1996). There is no suitable animal model available for the study of this disease. The severe combined immunodeficiency mouse model has been investigated recently, however tumours developing in the mice appear to be EBV-driven lymphoproliferations and not true HD lesions (Kapp *et al.*, 1993; Krajewski *et al.*, 1995). Recent studies using single RS cells micromanipulated from cell suspensions have provided information about gene expression in these cells (Trumper *et al.*, 1993), and should prove to be a useful future strategy in the investigation of HD.

Several well characterised oncogenes and tumour suppressor genes, including bcl-2 and p53, have been investigated with regard to HD and tumour development. Most of the studies investigating oncogene expression have been performed using HD-derived cell lines and these have an uncertain relationship to RS cells. The results are variable; expression of p53 has been found in a large proportion of HD cases, up to 90% (Neidobitek *et al.*, 1993; Dousis *et al.*, 1993). It has been suggested that p53 expression is inversely related to EBV LMP-1 protein expression (section 1.4.5.5). Chromosomal translocations involving bcl-2 in HD have been studied extensively in recent years with contradictory results. Some studies reported detection of bcl-2 translocations in a high proportion of HD cases and proposed that the translocation was important for tumorigenesis (Weiss *et al.*, 1987a; Stetler-Stevenson *et al.*, 1990; Bhagat *et al.*, 1993). Other studies, including unpublished findings from our group, did not find translocations which could be unequivocally demonstrated in the RS cells. The consensus at present is that bcl-2 does not play a central role in the development of HD; it is probable that the majority of bcl-2 translocations detected in biopsy material were present in the reactive tumour infiltrate and not the actual tumour cells.

The involvement of viruses in the aetiology of HD has been the focus of much investigation. A significant advance in the study of HD was the detection of EBV genomes in the RS cells in a significant proportion of cases (section 1.5.1). The potential role of other viruses e.g. HHV-6 has also been studied (section 1.5.2).

1.3 The Family Herpesviridae

The Herpesviridae are a family of double-stranded DNA enveloped viruses, members of which infect a diverse range of hosts, both human and animal (reviewed by Roizman & Sears, 1996). The genomes of these viruses are large, ranging from 125-229kb, with a coding capacity for over 100 genes. They are characterised by their ability to establish persistent infection by adopting a latent state.

1.3.1 Classification of herpesviruses

Herpesviridae are subdivided into three classes according to their biological properties; these are the alpha, beta and gammaherpesvirinae (Roizman, 1982; Roizman *et al.*, 1992). There are other classification systems in use, based on genome size and structure.

Alphaherpesvirinae are characterised by a short replicative cycle, productive infection of epithelial cells, and latency in sensory ganglia. Members of this group include herpes simplex viruses (HSV) types 1 & 2, Varicella Zoster virus (VZV) and equine herpesvirus (EHV) type 1. Betaherpesvirinae have slow replicative cycles and infected cells display cytomegaly. Secretory glands, kidney and lymphoreticular cells are among the tissues and cell types which can support latency *in vitro* (Britt & Alford, 1996). Members of this group include human cytomegalovirus (HCMV) and human herpesviruses (HHV) 6 & 7. Gammaherpesvirinae have lymphoid tropism and the site of latency is also in lymphoid tissue. This group includes Epstein-Barr virus (EBV) and herpesvirus Saimiri among its members.

Until recently, there were seven human herpesviruses which had been isolated and partially characterised (Table I.ii). In 1994 an eighth human herpesvirus was described (Chang *et al.*, 1994) and has been termed Kaposi's sarcoma associated herpesvirus (KSHV) or HHV-8. It has since been partially sequenced and characterised (Moore *et al.*, 1996), and viral particles have now been visualised (Said *et al.*, 1996).

1.3.2 Diseases associated with herpesvirus infections

Infection with a herpesvirus can result in a wide variety of different outcomes ranging from subclinical infection to extensive and sometimes fatal viraemia. Members of the herpesvirus family achieve lifelong infection of the natural host by establishing latency. Reactivation into productive infection from the latent state occurs in response to certain stimuli, e.g. psychological or physiological stress, exposure to UV light. Little is known about the mechanism of reactivation or the genes involved for different herpesviruses. The consequences of such reactivation are usually less severe than those of primary infection, with the exception of VZV (reviewed by Arvin, 1996), and are generally quickly and effectively controlled by the immune response of the host (Table I.ii).

Herpesviruses are of clinical importance as they can cause widespread viraemia in the immunocompromised host. HCMV can cause diffuse pneumonitis when reactivated in immunosuppressed patients such as HIV-infected individuals (Jacobson & Mills, 1988). It is also a major factor in the destruction and rejection of renal allografts and other transplanted organs, both during primary infection and on reactivation (Ho, 1982; Ruben *et al.*, 1985; Pollard, 1988). Perinatal infection with HSV-2 can produce fatal viraemia in the neonate where immunocompetence has not yet been fully established (reviewed by Whitley, 1996). EBV causes lympho-proliferative disorders and extensive lesions of the oral mucosa on reactivation in immunosuppressed patients (section 1.4.7).

The association of EBV with Burkitt's lymphoma (BL) (section 1.4.7.2) was the first instance that a virus had been implicated in the pathogenesis of a human cancer. The isolation of KSHV sequences from tissues of Kaposi's sarcoma, which is a neoplasm affecting AIDS sufferers (Chang *et al.*, 1994), could prove to be a firm link between this newest member of the herpesvirus family and a tumorigenic process.

Table I.ii Diseases associated with herpesvirus infection

virus	*main diseases caused*	
	immunocompetent host	*immunosuppressed host*
HSV-1	cold sores, eye infections encephalitis	
HSV-2	genital herpes	
VZV	chicken pox, shingles	
EBV	infectious mononucleosis; association with BL, nasopharyngeal carcinoma	lymphoproliferation
HCMV	congenital defects	pneumonitis, graft rejection
HHV-6	exanthem subitum	pneumonitis, encephalitis
HHV-7	exanthem subitum	
HHV-8/KSHV	*Kaposi's sarcoma ?body cavity-based lymphoma ?multicentric Castleman's disease	

* These diseases are more often seen in the context of HIV/AIDS-related immunosuppression but can occur in the immunocompetent host.

1.3.3 Replicative cycle of a herpesvirus

Herpesviruses have two distinct phases which are characteristic of their life cycle. These comprise a lytic and a latent phase. The lytic phase represents typical productive infection of permissive cell types and results in generation of progeny virions. The latent phase, occurring in non-permissive cells, enables the virus to persist undetected within the cell and express only a restricted number of viral genes.

1.3.3.1 Lytic infection

In order to gain entry into the cell the virus attaches to receptors on the target cell surface; expression of viral receptors by different cell types therefore has a major influence on the cellular tropism of individual herpesviruses. The virus penetrates the cell via interaction of spike glycoproteins in the viral envelope with cell surface molecules in the plasma membrane, causing fusion of the two membranes to occur (Roizman & Sears, 1996).

Once inside the cell the virus uncoats and the capsid is transported to the nucleus. Here the viral DNA is released and circularises. Transcription of the viral genome occurs, followed by translation of viral mRNA species into protein. At this stage, all host cell transcription and protein synthesis are shut down (Roizman & Roane, 1964). Translation of viral messages occurs in a temporal cascade with initial translation of viral transcription factors, followed by DNA modifying enzymes and finally the structural components of the virions. Viral DNA is replicated and packaged into capsids and as these exit the nucleus they acquire the viral envelope. The progeny virions are released from the cell in vacuoles and this process invariably results in the destruction of that cell (reviewed by Roizman & Sears, 1996).

1.3.3.2 Latent infection

Latent infection represents persistence of the herpesvirus within the host in a non-infectious state. Latency usually occurs in a cell type distinct from that in which lytic replication takes place. The viral genome exists as a closed episome, and only a restricted subset of viral genes are expressed (Roizman, 1996). This enables the virus to remain for years, avoiding detection by the host immune system.

1.4 Epstein-Barr virus

EBV was first isolated in 1964 by Epstein and co-workers from a culture of African BL cells (Epstein *et al.*, 1964). EBV is a gammaherpesvirus with a 172kb genome containing over 100 open reading frames (ORF). The prototype virus is B95-8 and the entire nucleotide sequence of this isolate has been determined (Baer *et al.*, 1984).

1.4.1 Infection with EBV

EBV is a ubiquitous virus with 90% of the adult population becoming seropositive in the first decade of life (reviewed by Evans & Niederman, 1989). Primary infection is almost always subclinical. Delayed infection, occurring at adolescence or later, gives rise to the clinical syndrome infectious mononucleosis (IM) in approximately 50% of cases (Henle *et al.*, 1968).

Transmission of the virus is by exchange of bodily fluids, principally saliva. Transmission by blood transfusion and bone marrow transplantation, and isolation of virus from vaginal secretions and cervical epithelial cells have all been reported (Henle & Henle, 1985; Gratama *et al.*, 1994; Sixbey *et al.*, 1986).

EBV gains entrance to B-lymphoid cells via attachment of the viral envelope glycoprotein gp340/220 to the CD21 (CR2) antigen on the cell surface (Nemerow *et al.*, 1985; 1987; reviewed by Nemerow *et al.*, 1990). Entrance to epithelial cells can occur by this method also (Young *et al.*, 1986; Sixbey *et al.*, 1987; Birkenbach *et al.*, 1992) or by IgA-mediated endocytosis in the case of otherwise resistant epithelial cells (Sixbey & Yao, 1992). Attachment is followed by fusion of the viral envelope with the plasma membrane. Infection of T-cells can also occur (Kikuta *et al.*, 1988). It has been suggested that this occurs via expression of the IFN-α receptor on these cells, as this receptor shares a common sequence motif with CD21 and is a member of the same family of receptors (Delcayre *et al.*, 1991; Watry *et al.*, 1991). IFN-α has been shown to prevent EBV from attaching to CD21 by binding to CD21 itself (Delcayre *et al.*, 1993).

1.4.2 Subtypes of EBV

EBV-1 and EBV-2, originally called subtypes A and B, are biologically distinct strains of the virus which can be distinguished on the basis of differences in several genes and their respective proteins (Sample *et al.*, 1990). Serology and virus isolation studies have shown that the serotypes have different distributions in nature, with EBV-1 being prevalent in developed countries such as Britain and North America, and EBV-2 being relatively more common in developing countries such as Africa and Papua New Guinea (Zimber *et al.*, 1986; Young *et al.*, 1987). It has been suggested that this distribution may display a bias due to the methods of detection used, and PCR based subtyping studies have since detected a more widespread distribution of type 2 virus in the West (Sixbey *et al.*, 1989).

1.4.3 Persistence of EBV in the infected host

Following primary infection with EBV, lifelong persistence of the virus results (Klein & Klein, 1985) and can be demonstrated in the following ways. First, continuous EBV-transformed B-cell lines can be grown in culture from the peripheral blood of infected donors (Rickinson *et al.*, 1984). Secondly, infectious virus can be recovered from throat washings of healthy seropositive individuals years after primary infection (Yao *et al.*, 1985a).

In the 1980s the site of initial viral replication following primary infection was believed to be the mucosa of the buccal cavity and oropharynx, e.g. salivary gland and pharyngeal epithelium. These cells are permissive for the virus and replication at these sites is productive; infectious virus is shed in the saliva (Yao *et al.*, 1985a). B-cells circulating through the oropharynx were thought to become infected by the virus produced by epithelial cells at these sites. Recent opinion is that B-lymphoid tissue, such as the tonsils, is the site of primary EBV infection and that infection of proximal epithelial cells is a secondary event (Neidobitek *et al.*, 1992; Neidobitek & Young, 1994).

There has been much discussion regarding the true sites of latency and productive infection; the current consensus favours the idea that lymphoid tissue, rather than epithelium, is the site of latency for three main reasons. First, treatment with antiviral compounds, such as acyclovir, results in a reduction in the amount of virus shed in the saliva, but has no effect on the

number of circulating lymphocytes which are EBV-positive (Yao *et al.*, 1989b). This implies that lytic cycle replication, the target for acyclovir, occurs in the oropharyngeal mucosa. Secondly, oral hairy leukoplakia (OHL) (section 1.4.7.1), which occurs in the epithelium of the tongue in immunosuppressed people, is characterised by lytic phase EBV replication with no detectable latent phase (Greenspan *et al.*, 1985), suggesting that epithelial cells are permissive for viral replication and cannot support the latent phase. Thirdly, in bone marrow transplant recipients whose own marrow has been irradiated, eradication of EBV infection has been recorded (Gratama *et al.*, 1988). This would suggest that cells of a haemopoietic derivation are the site of true viral latency.

The proposed site of viral persistence in the infected host is therefore lymphoid tissue. The role of nasopharyngeal epithelium as the main site for production of infectious virus has recently been challenged by the results of a study of IM patients (Anagnostopoulos *et al.*, 1995). Both latent and productive infection were demonstrated in lymphoid cells of the tonsillar crypts and epithelium, whereas no productive replication was detected in the epithelial cells. A second study of EBV seropositive people in southern China, without EBV-related disease, showed productive infection occurring in mucosal lymphocytes but no such infection of the nasopharyngeal epithelial cells (Tao *et al.*, 1995). This would suggest that lymphoid tissue could be the site of productive replication in addition to being the principle reservoir of latent viral genomes in the infected host, with infected epithelium acting as a secondary site of viral shedding.

The study of the association between nasopharyngeal carcinoma (NPC) and EBV provides evidence for EBV adopting a latent, rather than purely lytic, state in epithelial cells. These tumours are of epithelial origin and the virus is clearly present in the latent state with expression of several viral antigens (Brooks *et al.*, 1992). This may, however, still be reconcilable with a B-cell site for true latency. EBV may initially productively infect the nasopharyngeal epithelium, which fits with the model for latent and lytic sites described above. This would correlate with the increased serum titres to lytic cycle antigens which precede the onset of NPC tumours (section 1.4.7.3). A subset of epithelial cells may then aberrantly enter the latent phase, thus achieving a proliferative advantage and increasing the probability of progression to NPC.

1.4.4 EBV infection *in vitro*

EBV is unique in its ability to efficiently immortalise infected B-lymphocytes in culture. In a cell culture situation, 10-100% of B-cells successfully infected with EBV will go on to yield progeny that can proliferate indefinitely, maintaining EBV within the cells in the latent state (Sugden, 1989). Infection of peripheral blood mononuclear cells (PBMCs) with EBV is the standard means of establishing a continuous human cell line from a specific donor.

Latent EBV can be stimulated to productive replication using 12,0-tetradecanoyl phorbol-13-acetate (TPA) (Table II.ii). In a continuous EBV-driven culture, one in 10^2-10^6 cells will undergo spontaneous reactivation at any one time (Sugden *et al.*, 1979). This occurs through triggered expression of the BZLF-1 gene, possibly involving EBNA-1, which activates the lytic gene expression cascade (reviewed by Miller, 1990).

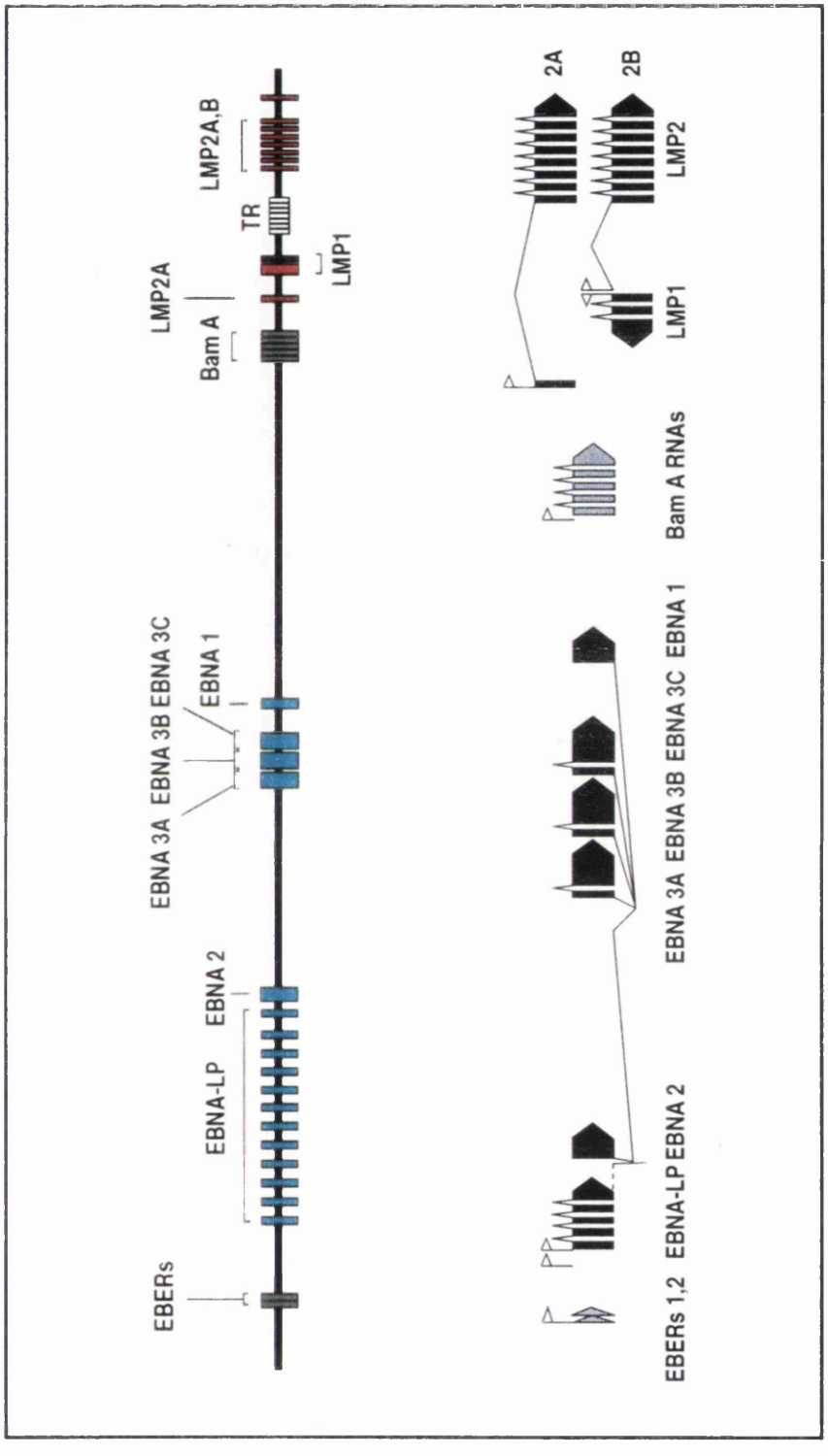
1.4.5 EBV latent proteins

Latent infection *in vitro* results in immortalisation and activation of the host B-cell. The resulting immortalised cell line is known as a B-lymphoblastoid cell line or LCL (Pope *et al.*, 1968), and gene expression in these cells is restricted to a small subset of viral genes known collectively as the latent genes. Much study has gone into this phenomenon, unique among the herpesviruses, with emphasis on the functions of the individual gene products (Kieff & Leibowitz, 1990; Ring, 1994).

In an LCL *in vitro*, the virus expresses a number of latent genes. These include the Epstein-Barr viral nuclear antigens (EBNAs) 1, 2, 3a, 3b, 3c, leader protein (EBNA-LP); the latent membrane proteins LMP-1 and LMP-2a and -2b and the Epstein-Barr encoded RNAs (EBERs) (reviewed by Sugden, 1994). These genes are expressed in various combinations during latency, except in the case of EBNA-1, the expression of which is obligatory. There is also expression of *Bam*HI A fragment RNA transcripts during latency but little is known about their function (section 1.4.6). The location and orientation of the latent genes as they are expressed from the EBV episome is illustrated in Fig.1.4.

Fig1.4 Transcripts are shown in the correct orientation relative to one another. Promoters are marked by pennants and splicing patterns are indicated. Exons for non-coding RNAs are shown in grey and for mRNAs in black. The actual genomic positions of these exons are given (top) on a linear map of the EBV genome. Note that the genome is circularised in latency due to fusion of the terminal repeats (TR); the LMP-2 transcripts are spliced across this region.

Fig.1.4 Orientation and expression of EBV latent genes



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1.4.5.1 EBNA-1

Expression of EBNA-1 during latency ensures that the viral genome is replicated and maintained as an episome within the infected cell (Yates *et al.*, 1984; 1985). The EBNA-1 ORF encodes a relatively small protein of 641 amino acids (aa) which has three distinct domains. The carboxy terminal domain has DNA binding activity, there is a central domain containing glycine-alanine repeat sequences and the amino terminal third is highly hydrophilic (Hennessy & Kieff, 1983; Ambinder *et al.*, 1991). The DNA binding domain transactivates OriP, the plasmid origin of replication (Ambinder *et al.*, 1990). EBNA-1 also regulates its own expression (Reisman & Sugden, 1986). It has been shown to associate with chromatin and the nucleoplasm in latently infected cells. Oncogenic properties have recently been attributed to this gene following studies using EBNA-1 transgenic mice (Wilson & Levine, 1992; Wilson *et al.*, 1996).

1.4.5.2 EBNA-2

EBNA-2 is a transactivator of both cellular genes and viral latent genes, including its own gene and that of LMP-1 (Wang *et al.*, 1990). EBNA-2 is one of the first viral genes to be expressed in newly infected B-cells *in vitro*. The gene is thought to be essential for the *in vitro* immortalisation function of EBV (Hammerschmidt & Sugden, 1989; Cohen *et al.*, 1989; 1992). A mutant strain of EBV, P3HR-1, which has a deletion of EBNA-2 and partially EBNA-LP, is transformation incompetent (Bornkamm *et al.*, 1982). Reconstitution of an intact EBNA-2 gene restores this function. EBNA-2 transfected into Rat-1 cells cannot transform the cells but does cause reduced serum dependence (Dambaugh *et al.*, 1986). EBNA-2 also induces high levels of expression of the CD23 activation marker (Wang *et al.*, 1987) and causes BL cells to grow in tight clumps as opposed to single cells.

As outlined above, there are two subtypes of EBV, type-1 and type-2 (section 1.4.2). The subtypes differ in the EBNA-2 gene among others (Aldlinger *et al.*, 1985), and also differ in their growth transformation properties. EBV-1 transforms B-cells in culture more efficiently than EBV-2 (Rickinson *et al.*, 1987).

1.4.5.3 The EBNA-3 family

The EBNA-3 genes a, b and c have a common origin and are tandemly located along the EBV genome. Of these genes, EBNA-3a and -3c are essential for viral transformation of B-cells (Tomkinson & Kieff, 1992; Tomkinson *et al.*, 1993); EBNA-3c may ensure high levels of LMP-1 are maintained in proliferating EBV-infected B-cells (Allday & Farrell, 1994). There are noticeable similarities between these proteins even though the amino acid sequences are not closely conserved. The proteins are distributed similarly throughout the nuclei of latently infected cells, being associated with the nuclear matrix but not strongly associated with chromatin (Petti *et al.*, 1990). The genes coding for the EBNA-3 proteins differ between the two subtypes of EBV (Sample *et al.*, 1990).

1.4.5.4 EBNA-LP

Leader protein (EBNA-LP) is so called because it is encoded by the leaders of the other EBNA mRNAs and translated when these messages are spliced. The protein is one of the first to be detected in the newly infected cell, alongside EBNA-2, but is not essential for immortalisation (Hammerschmidt & Sugden, 1989). It is believed, however, to fulfil an important auxiliary role because deletion mutants are greatly impaired in their transformation efficiency (Hammerschmidt & Sugden, 1989; Mannick *et al.*, 1991). Recent studies have discovered colocalisation of EBNA-LP with the retinoblastoma protein (Rb) within the nucleus. This is comparable to the distribution of SV40 large T antigen and Rb (Jiang *et al.*, 1991), with complex formation resulting in inactivation of Rb. The EBNA-LP protein structure shows some similarity to E1A of adenovirus 5, another transforming protein shown to complex with Rb. These findings support the theory that EBNA-LP plays an important, albeit not essential, role in cellular growth transformation by EBV. In addition, it is thought that EBNA-LP could play a role in the complex mechanisms involved in EBV RNA processing and splicing due to its distinct nuclear distribution into discrete, sub-nuclear particles (Wang *et al.*, 1987; Petti *et al.*, 1990).

1.4.5.5 The latent membrane proteins

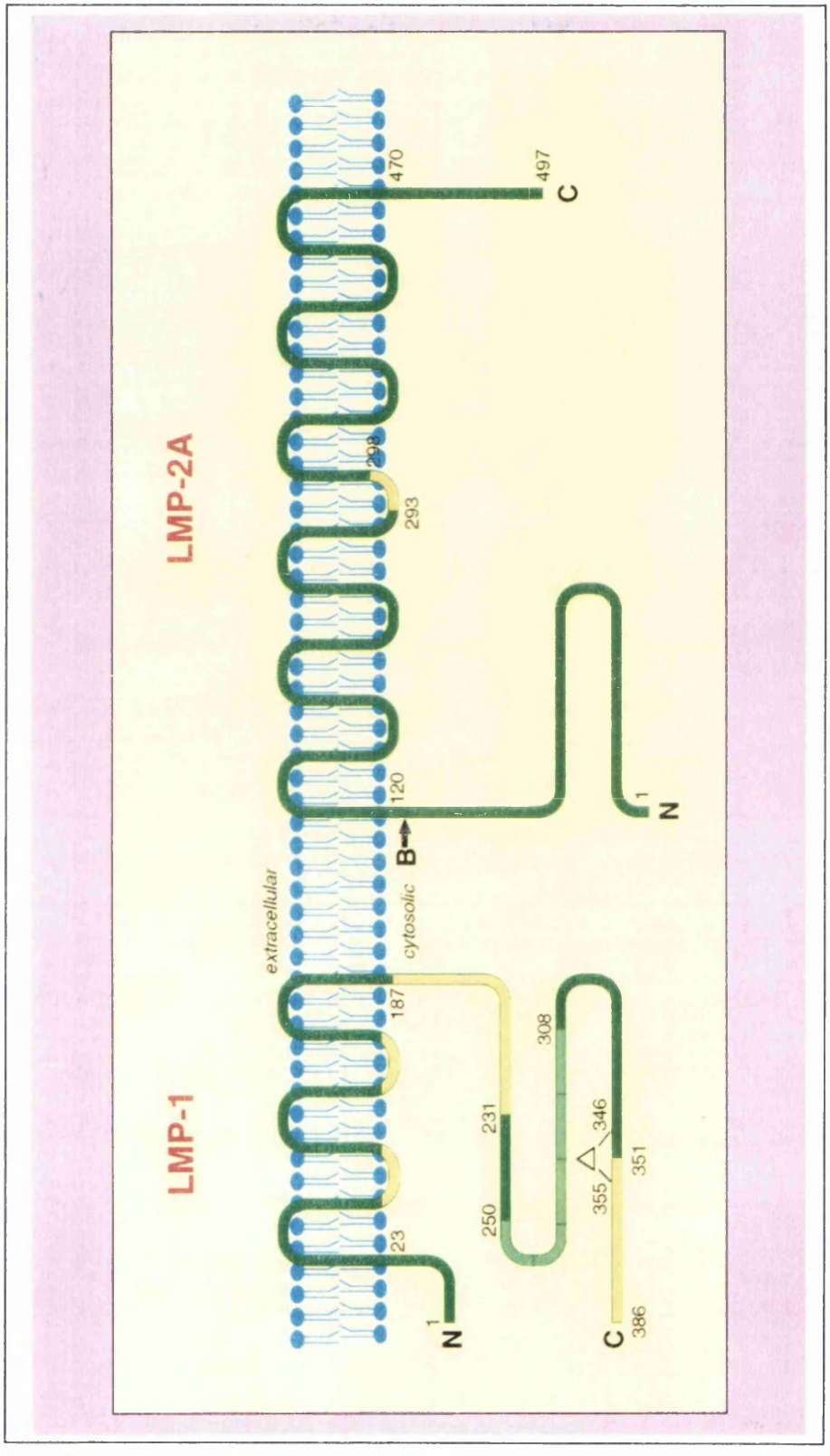
The LMP-1 gene has a potentially oncogenic function in the latently infected cell. This viral gene behaves as a classical oncogene in tissue culture assays, transforming Rat-1 cells *in vitro* (Dambaugh *et al.*, 1986; Moorthy & Thorley-Lawson, 1993). This results in altered morphology and decreased serum requirement of the cells (Dambaugh *et al.*, 1986). Loss of contact inhibition and reduced anchorage-dependent growth are observed in LMP-1 transfectants (Wang *et al.*, 1985; Baichwal & Sugden, 1988). LMP-1 plays a role in immortalisation of B-cells in culture; genetically modified mutants lacking full length LMP-1 are transformation deficient (Kaye *et al.*, 1993; 1995). Expression of LMP-1 *in vitro* induces increased expression of cellular activation markers LFA-1 and LFA-3 and the cellular adhesion molecule ICAM-1. It has also been shown to increase levels of bcl-2 and suppress apoptosis (Henderson *et al.*, 1991; Rowe *et al.*, 1994).

The molecule has a similar structure to the G-protein-coupled receptor family and forms discrete patches in the cell membrane (Liebowitz *et al.*, 1986). The predicted topology of LMP-1 is shown in Fig.1.5. The protein is thought to modify signal transduction pathways and has been shown to act in the TNF-α signal cascade (Arvanitakis *et al.*, 1995; Mosialos *et al.*, 1995), specifically inducing expression of the epidermal growth factor receptor (Miller *et al.*, 1995). It has also been shown to activate the NF-κB transcription factor (Herrero *et al.*, 1995; Huen *et al.*, 1995).

LMP-1 sequence variants, which may have altered biological properties, have been found in certain populations. Common variations include loss of an *Xho*I restriction site at the N-terminal end and deletions in the C-terminal region of the protein (Fig.1.5); these have been found in Southern Chinese and Alaskan Inuit populations (Lung *et al.*, 1990; Hu *et al.*, 1991; W.Miller *et al.*, 1994). These mutations are found in EBV isolates from NPC cases, and similar deleted forms of LMP-1 have been isolated from BL tumours (Chen *et al.*, 1996). These links between LMP-1 deletion mutants and malignancy have led to the suggestion that they may represent more oncogenic forms of the protein. Some variants have been shown to have greater transforming properties *in vitro* (Chen *et al.*, 1992), have decreased sites for phosphorylation (W.Miller *et al.*, 1994), and be less immunogenic in some systems (Trivedi *et al.*, 1994). The latter observation raises the possibility of non-immunogenic LMP-1 escape mutants arising under immunoselection pressure (section 1.7.3). Deleted LMP-1 species are also found in EBV isolates from HD lesions, and these are identical to those found in NPC (Knecht *et al.*, 1993; 1995).

Fig.1.5 Schematic representation of LMP-1 and LMP-2a. The triangle indicates the deletion common to many LMP-1 variants. Functional domains affecting NF-κB activation and transformation are shown in yellow. LMP-2A is shown on the right, with the start codon of LMP-2b indicated by the arrow. The yellow domain represents a potential ATP-binding motif (Longnecker *et al.*, 1991).

Fig.1.5 Predicted topology of the LMPs



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The LMP-2s are two similar proteins which are transcribed across the terminal repeat sequences following circularisation of the viral genome (Laux *et al.*, 1988; reviewed by Longnecker, 1994). Transcription of the LMP-2 genes occurs in the opposite direction to transcription of LMP-1. LMP-2b is an N-terminally truncated form of LMP-2a; the proteins are 40kD and 54kD respectively and both colocalise to the plasma membrane alongside LMP-1 (Longnecker & Kieff, 1990). The predicted structure of LMP-2 is shown in Fig.1.5. The LMP-2s may modulate the activation effects of LMP-1 and be important for stabilising the latent state (C.Miller *et al.*, 1994). Both LMP-2s are dispensable for B-cell transformation *per se* (Longnecker *et al.*, 1992; 1993a; 1993b) but are thought to contribute to the infected cell phenotype as indicated by their expression in several malignancies such as HD and NPC.

1.4.5.6 EBERs

EBER-1 and EBER-2 are small nonpolyadenylated, untranslated RNA transcripts which are present in very high copy numbers, approximately 10^5-10^7 per cell, within the nucleus of the latently infected cell (reviewed by Clemens, 1994). Their function is as yet unknown but appears non-essential for the transformation process (Swaminathan *et al.*, 1991). Currently, the main interest in the EBERs is as a target for probes used in *in situ* hybridisation.

1.4.6 Patterns of EBV latent gene expression

Three distinct patterns of EBV latent gene expression have been described for infected B-cells *in vitro*. These are designated latency (Lat) I, II and III (Rowe *et al.*, 1992) and are outlined in Table I.iii. Lat I is seen in the tumour cells of fresh EBV-positive BL biopsies and some BL-derived cell lines (Rowe *et al.*, 1987). Lat II expression is characteristic of RS cells of EBV-associated HD (Pallesen *et al.*, 1991; Deacon *et al.*, 1993; Grasser *et al.*, 1994) and tumour cells of undifferentiated NPC (Young *et al.*, 1988; Brooks *et al.*, 1992). The Lat III pattern, with unrestricted expression of all the latent gene products, is seen in cultured LCLs *in vitro* and some long-term cultured, EBV-positive, BL-derived cell lines. In all known forms of latency there is expression of the EBERs and also latency-associated RNA transcripts expressed from the *Bam*HI A fragment (Gilligan *et al.*, 1991; Brooks *et al.*, 1993).

Table I.iii Patterns of EBV latent gene expression

latency	*viral/cellular gene expression*	*cells expressing this type of latency*
Lat I	EBNA-1 only CD10, CD77	1° BL cells group I BL-derived cell lines
Lat II	EBNA-1, LMP-1, LMP-2 (CD10, CD77) (CD21, CD23, CD30)	RS cells of HD, NPC tumour cells group II BL-derived cell lines
Lat III	all EBV latent genes CD21, CD23, CD30 LFA-3, ICAM-1	LCLs, EBV-driven LPD in immunosuppressed people group III BL-derived cell lines

*Antigens given in brackets are not expressed in all cases of Lat II, e.g. they are not present on RS cells of HD.

In addition to expression of EBV latent genes, the Lats are characterised by differences in expression of cell-surface markers such as cellular activation antigens and adhesion molecules. Lat I is associated with expression of CD77 (BL-associated antigen) and CD10 only (Rooney *et al.*, 1986; Gregory *et al.*, 1988). In Lat II, additional expression of one or more activation markers, e.g. CD21, CD23 and CD30, is seen (Rowe *et al.*, 1992). Lat III is characterised by expression of all the activation markers plus cell adhesion molecules such as LFA-3 and ICAM-1, with loss of expression of CD10 and CD77 (Rowe *et al.*, 1992).

In the *in vivo* situation in healthy EBV-positive individuals, the pattern of latent gene expression in infected cells is distinct from the expression patterns described above. It is characterised by expression of EBNA-1 and LMP-2 only, as determined by analysis of mRNA from infected cell populations (Qu & Rowe, 1992; Tierney *et al.*, 1994; Chen *et al.*, 1995). The infected cells may also be phenotypically distinct (Gregory *et al.*, 1987; Miyashita *et al.*, 1995).

1.4.7 Disease associations

EBV has been implicated in the aetiology of many lymphoid and epithelial conditions, including several malignancies. It is difficult experimentally to establish a firm relationship between a virus and a human malignancy. Association with a virus can be proposed on the basis of epidemiological and molecular findings. In the case of EBV, which is carried for life by 90% of the world's population, association must be clearly demonstrated.

It is thought that the proliferative potential of EBV-infected cells, coupled with lifetime exposure through viral persistence, are major predisposing factors in the development of EBV-related neoplastic disease.

1.4.7.1 Benign diseases associated with EBV

IM is characterised by a polyclonal B-cell proliferation. T-cell responses to this proliferation are largely responsible for the clinical manifestations of this syndrome and for the atypical lymphocytosis. It is a self-limiting disease which usually resolves in time, however it can give rise to a chronic disease.

OHL occurs in immunocompromised individuals, most notably in HIV-infected people (Greenspan *et al.*, 1984) and less commonly in post-transplant patients (Macleod *et al.*, 1990). OHL presents as a raised, "hairy" white lesion occurring on the lateral borders and/or ventral surfaces of the tongue (Schiodt *et al.*, 1987). It is associated with chronic productive EBV infection of the upper epithelial cells of the tongue (Niedobitek *et al.*, 1991). Lytic phase EBV has been detected in tissue sections using immunohistochemistry and *in situ* hybridisation, and more recently PCR (Mabruk *et al.*, 1994).

1.4.7.2 Burkitt's lymphoma

BL is a malignancy of B-cell origin. There are two forms of the disease, endemic and sporadic. The endemic type shows high incidence in parts of equatorial Africa and Papua New Guinea, and the sporadic form of the disease has 100-fold lower incidence worldwide (Magrath, 1990). The two forms of BL are histologically very similar but display different clinical presentation and aetiology.

Endemic BL, which occurs primarily in children, is associated with EBV in virtually 100% of cases, with viral genomes being present in all the tumour cells and these genomes being clonal (Epstein & Achong, 1979). The pathogenesis of endemic BL is thought to involve a number of events which include virus-induced B-cell proliferation. The fully malignant BL phenotype requires the presence of a chromosomal translocation which brings about dysregulation of the c-myc gene by juxtaposing this gene to an immunoglobulin gene locus (Dalla-Favera *et al.*, 1982; Klein & Klein, 1985). There are three possible translocations which can achieve this result; 8:14 (IgH locus), 8:22 (λ light chain) or 2:8 (κ light chain). Sporadic BL tumours also possess the c-myc translocation but are associated with EBV in only 10-20% of cases. The occurrence of holoendemic malaria is thought to facilitate tumorigenesis in EBV-associated endemic BL, although the exact mechanism by which this exerts its effect is unknown. High titres to EBV antigens precede the onset of the tumour by several months.

1.4.7.3 Nasopharyngeal carcinoma

NPC is endemic in Southern China where it is the most common cancer of men and second most common cancer of women (Zur Hausen *et al.*, 1970). The undifferentiated form of the disease does not occur in EBV seronegative individuals and displays complete correlation with presence of the viral genome in the tumour cells (Raab-Traub *et al.*, 1987); indeed this malignancy shows the most consistent world-wide association with EBV of all malignancies associated with this virus. The tumour cells of NPC contain multiple EBV genomes and express viral antigens (Fahraeus *et al.*, 1988; Young *et al.*, 1988). This strongly suggests a causal role for the virus in tumourigenesis, although the restricted geographical and ethnic incidence of the disease (de The, 1982) would indicate that EBV infection, although necessary, is not sufficient for tumour development. Studies have been carried out to investigate the existence of genetic and environmental co-factors. Dietary factors such as foods which are high in nitrosamines and phorbol esters have been implicated (Zou *et al.*, 1994).

Serologically, high antibody titres to EBV VCA and EA occur in NPC patients. Of particular interest is the finding that elevated IgA titres to these viral antigens precede the onset of clinical disease by several years; the elevation of antibody titres also has prognostic significance, with higher titres indicating high tumour load and poor prognosis (Henle & Henle, 1976; Yip *et al.*, 1996).

1.4.7.4 Hodgkin's disease

HD has been associated with EBV in approximately 40% of cases. A more detailed account of this association is given in section 1.5.1.

1.4.7.5 B-lymphoproliferative disease

EBV-induced B-lymphoproliferative disease (LPD) is observed among immunosuppressed individuals, both in the case of congenital and acquired immunosuppression (Sullivan & Woda, 1989). Groups of individuals where there are well documented reports of LPD include post transplant patients (Shapiro *et al.*, 1988) and HIV-positive people suffering from AIDS (Hamilton-Dutoit *et al.*, 1991). Cases of HD arising in these people also tend to be EBV-positive (Hamilton-Dutoit *et al.*, 1991). LPD is thought to arise from B-cells expressing all the EBV latent proteins; such a cell would normally be expected to be lysed by the host cellular immune response, but in the immunosuppressed person is allowed to persist. Where this occurs, viral antigens normally only transiently expressed in the immunocompetent host are constitutively expressed in the infected cell. Several of these proteins have been shown to play a role in growth transformation, and it is thought that the action of these viral gene products leads to increased proliferation and survival of the infected cells. This is believed to be the initiating step in the development of malignant lymphoma, but the exact mechanism of this progression is not understood. In cases where LPD has progressed to a clonal cell population, restoration of immune function can still result in complete regression of the lesion (Starzl *et al.*, 1984). This is possibly the only example of a virally associated tumour where the virus is solely responsible for the initiation and maintenance of the neoplastic state.

1.5 Viral Involvement in HD

The epidemiological features of HD discussed in section 1.2.3 suggest an infectious aetiology with the strongest evidence relating to the young adult age group. Attempts have been made to correlate the incidence of HD with infection with several known viruses, particularly those associated with common childhood infections.

1.5.1 EBV

Serological studies have investigated potential associations between HD and antibody titres to a number of viruses, mainly herpesviruses. The most consistent correlation was found for EBV. Studies in the 1970s reported raised antibody titres to EBV in HD patients. This suggested an association between the virus and the disease (Levine *et al.*, 1971; Henle & Henle, 1973; Langenhuysen *et al.*, 1974; Hesse *et al.*, 1977; Evans *et al.*, 1978). It was later found that individuals with a past history of IM, a condition associated with delayed primary infection with EBV, were three times more likely to develop HD than those with no such history (Munoz *et al.*, 1978; Gutensohn & Cole, 1980). An investigation was carried out by Mueller *et al.* in 1989 to determine whether the raised antibody titres to EBV preceded diagnosis. A group of 43 HD patients, whose serum had been stored prior to diagnosis of the disease, were studied alongside 96 controls (Mueller *et al.*, 1989). The data indicated a significantly increased relative risk of developing HD in individuals with raised antibody titres. These results suggest that the elevation of antibody titres to EBV seen in HD patients is not simply due to viral reactivation induced by therapy or advanced disease state. The findings, together with the data on IM and HD, strengthened the evidence that EBV is involved in the pathogenesis of this disease.

Early molecular studies were undertaken to determine whether EBV had a causal role in the disease, and as no detectable viral genomes were found in the disease lesions it was believed that this was not the case (Pagano *et al.*, 1973; Lindahl *et al.*, 1974). These studies included only small numbers of cases and the sensitivity of the techniques used in the analyses, which utilised either radiolabelled virus or cRNA as a probe, was poor by present day standards. It was not until much later that it was demonstrated by Weiss *et al.* (1987b) that EBV genomes were detectable by Southern blot

analysis in 4 of 21 HD cases (Weiss *et al.*, 1987b). This study utilised the *Bam*HI W fragment as a probe (Arrand *et al.*, 1981) and this proved more sensitive as this is a repeat element. Since that time studies using this technique and others have shown EBV genomes to be present in HD samples from 17-50% of cases (Anagnostopoulos *et al.*, 1989; Jarrett *et al.*, 1991; Weiss *et al.*, 1991; P.G.Murray *et al.*, 1992; Herbst *et al.*, 1992; Khan *et al.*, 1993a; Poppema & Visser, 1994a; Belkaid *et al.*, 1995). The results of these studies are summarised in Table I.iv.

By examining the terminal repeat sequences of the viral genomes present in any given infected cell population it is possible to assess the clonality of the virus (Raab-Traub & Flynn, 1986). In the above studies the EBV genomes found in HD samples were shown to be of a clonal nature. Jarrett *et al.* (1991) examined tumour material from 27 cases of HD which were positive by Southern blot analysis and found that in all cases the EBV was clonal (Jarrett *et al.*, 1991; Armstrong *et al.*, 1993).

The central question to be addressed was whether the EBV genomes could be localised to the tumour cell population as opposed to the bystander lymphocytes in the tumour mass. EBV was detected in cells with RS-like morphology using DNA *in situ* hybridisation to the *Bam*HI W region (Weiss *et al.*, 1989). Immunohistochemical assays for LMP-1 showed EBV gene expression which localised to RS cells (Pallesen *et al.*, 1991; Armstrong *et al.*, 1992; P.G.Murray *et al.*, 1992). The EBERs, two small nonpolyadenylated RNA transcripts, are also expressed. *In situ* hybridisation to the EBERs is a very sensitive indicator of EBV infection due to the high copy number of EBER transcripts present in the nuclei of latently infected cells. EBER *in situ* hybridisation localised to the RS cells in 30-50% of cases and was found to be much better for detecting EBV in RS cells than any other method. With regard to the methods described above, EBER *in situ* hybridisation is widely recognised as the most useful for defining EBV status in HD (Weiss *et al.*, 1991; Armstrong *et al.*, 1992; 1994; Herbst *et al.*, 1992). Studies determining cellular localisation also demonstrated that RS cells express a pattern of latent genes that includes EBNA-1, LMP-1 and LMP-2 (Pallesen *et al.*, 1991; Deacon *et al.*, 1993; Grasser *et al.*, 1994).

Table I.iv Results of studies detecting EBV in HD cases

investigators	*year*	*country*	*detection method used*	*proportion of EBV+ cases*
Anagnostopolous *et al.*	1989	Germany	S.blot B*am*HI W *in situ* B*am*HI W	17%
Jarrett *et al.*	1991	UK	S.blot B*am*HI W	36%
Weiss *et al.*	1991	USA	PCR EBNA-1 *in situ* EBER	48%
Murray *et al.*	1992	UK	APAAP LMP-1	48%
Herbst *et al.*	1992	Germany	APAAP LMP-1 *in situ* EBER	50%
Khan *et al.*	1993	UK	*in situ* EBER	32%
Poppema & Visser	1994	Canada	*in situ* EBER APAAP LMP-1	26%
Belkaid *et al.*	1995	France	APAAP LMP-1 *in situ* B*am*HI W *in situ* EBER PCR B*am*HI W	38%

S.blot B*am*HI W	Southern blot using probes to B*am*HI W repeat region of EBV
in situ B*am*HI W	*in situ* hybridisation using probes to B*am*HI W repeat region of EBV
APAAP LMP-1	Alkaline phosphatase anti-alkaline phosphatase immunohistochemical staining using antibodies to EBV LMP-1
in situ EBER	*in situ* hybridisation using probes to EBV EBERs
PCR B*am*HI W	PCR using primers specific for B*am*HI W internal repeats
PCR EBNA-1	PCR using primers specific for 80bp of EBNA-1 gene

The heterogeneous nature of HD has led to the investigation of EBV-association within different subgroups of HD. Several studies have addressed the association of EBV with the histological subtypes of HD (section 1.2.1). The results of these studies find a significant association between EBV and HDMC; 50-90% of cases are EBV-associated. There is a much weaker association between EBV and HDNS, with only 10-50% of cases being EBV-associated (Pallesen *et al.*, 1991; Weiss *et al.*, 1991; Delsol *et al.*, 1992; Khan *et al.*, 1993a). HDLP cases are generally EBV-negative and it is likely that HDLP, as recently defined, is an EBV-negative disease. HDLD cases are usually EBV-associated, though the number of cases that have been studied is small.

Association of EBV with different age groups of cases has also been investigated. Paediatric HD cases (0-9 years) and older adult age group cases (over 50 years) were more often EBV-associated, with a dearth of such cases in the young adult (15-24 years) age group (Armstrong *et al.*, 1993; Jarrett *et al.*, 1996) A comparison of the proportion of EBV-associated cases in children in developing countries with young adults in developed countries does not support the polio model as described in section 1.2.3.1 (Armstrong *et al.*, 1993). At present the multiple aetiology hypothesis is more likely to relate to HD.

It is now almost certain that EBV plays a direct role in the development of a subgroup of cases of HD, although the mechanism of action of the virus remains unclear. In non-EBV-associated cases there may be another virus involved which acts in a similar manner within the cell and therefore results in the same clinical manifestations. This is in keeping with the widely acknowledged heterogeneous nature of HD.

1.5.2 HHV-6

The virus was first isolated in 1986 from patients with lymphoproliferative and immunosuppressive conditions (Salahuddin *et al.*, 1986). It is now known to be a ubiquitous virus with seroconversion occurring in early life and a persistent infection being established (Jarrett *et al.*, 1990). A number of studies have been undertaken to investigate HHV-6 antibody status in HD (Ablashi *et al.*, 1988; Biberfeld *et al.*, 1988; Clark *et al.*, 1990; Torelli *et al.*, 1991), one of these being a large case controlled study covering various lymphoid malignancies (Clark *et al.*, 1990). All found elevated antibody

titres to HHV-6 in HD. Raised titres were most marked in the young, as compared to older adult cases (Clark *et al.*, 1990). EBV serology carried out on the same serum samples indicated that the antibody profiles of the two viruses are largely independent of each other (D.Clark, unpublished results). The raised titres suggested the possibility of involvement of HHV-6 in HD. However, no viral genomes were detectable by Southern blot analysis in any of over 50 cases studied by our group and this would indicate that HHV-6 is unlikely to play a direct role in pathogenesis (Gledhill *et al.*, 1991; R.Jarrett, personal communication).

Since the latter analyses there have been reports of detection of HHV-6 DNA in HD cases by PCR and Southern blot analysis (Torelli *et al.*, 1991). However these studies found HHV-6 in only 3 of 65 cases (Torelli *et al.*, 1991) and the virus has not been localised to the tumour cells (Khan *et al.*, 1993a). Despite the results from these molecular analyses, the serological data do indicate that this virus could be associated with the young adult cases (Clark *et al.*, 1990). Further studies, such as more sensitive *in situ* hybridisation or single cell PCR, need to be undertaken to determine the role, if any, played by HHV-6 in these cases.

1.5.3 Other viruses in Hodgkin's disease

The epidemiological features of HD (section 1.2.3) suggest that the young adult cases, in particular young adult HDNS cases, are most likely to be linked with an infectious agent. To date, no one infectious agent has been identified which could account for the young adult peak of incidence. EBV is associated with a significant proportion of cases in other age groups, but there is a marked lack of EBV-associated cases in the young adult peak. HHV-6 has intriguing seroepidemiology, but no evidence exists for a direct role for this virus in the development of the disease.

There is much interest at present in the possible isolation of another infectious agent in these cases, especially a common virus which establishes persistent infection following primary exposure. Various molecular techniques are currently being employed to search for both known and novel viral sequences in the tissues of HD patients. Studies were carried out by our group to look for adenovirus serotypes 5 and 12, lymphotropic papovavirus and HHV-7 and -8. The results of these studies have so far been negative (A.Armstrong, personal communication).

1.6 Immunity and the MHC

The major histocompatibility complex (MHC), also referred to in humans as the human leukocyte antigen (HLA) region, has been intensively studied over the past two decades especially in relation to the disease associations of specific HLA types (Lechler, 1994). The MHC region is most extensively characterised in man and mouse and is a highly conserved genetic locus encoding approximately 200 genes. The locus is present on chromosome 6 in humans and includes the genes which code for the MHC class I and class II molecules (Fig.1.6). The MHC genes are the most polymorphic set of mammalian genes identified to date.

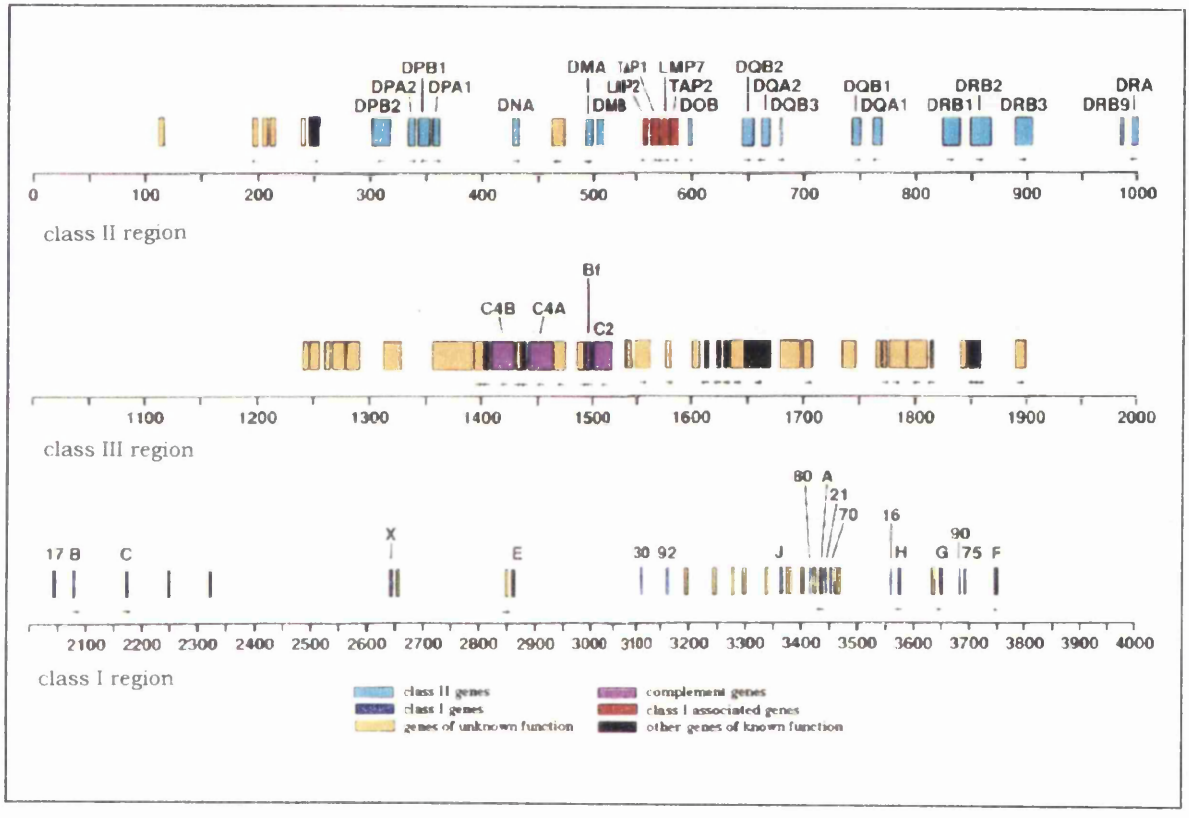
1.6.1 MHC class I

Most nucleated cell types express MHC class I molecules. The class I antigen presentation system allows any protein species present within a cell to be processed and represented at the cell surface in conjunction with MHC class I molecules as part of a random sampling of the cell cytoplasm. In this way the cell presents peptides which are representative of its internal environment to the CD8+ cytotoxic T-lymphocyte (CTL) compartment of the immune system. Thus, MHC class I molecules present endogenous peptides. There are three groups of MHC class I molecules and these are encoded by the HLA-A, -B and -C genes.

1.6.1.1 Structure of MHC class I molecules

In 1987 the crystal structure of the class I molecule was resolved (Bjorkman *et al.*, 1987a; 1987b). The molecule exists as a heterodimer comprising a heavy chain, or α-chain, and a light chain known as $\beta_2$microglobulin (β_2m). The α-chain has three domains (Fig. 1.7a); α_1 and α_2 form two α-helices either side of a β-pleated sheet and this represents the peptide binding groove as illustrated in Fig.1.7b (section 1.6.1.2). The α_3 domain and β_2m form anti-parallel β-pleated sheets and make up the part of the molecule more proximal to the plasma membrane.

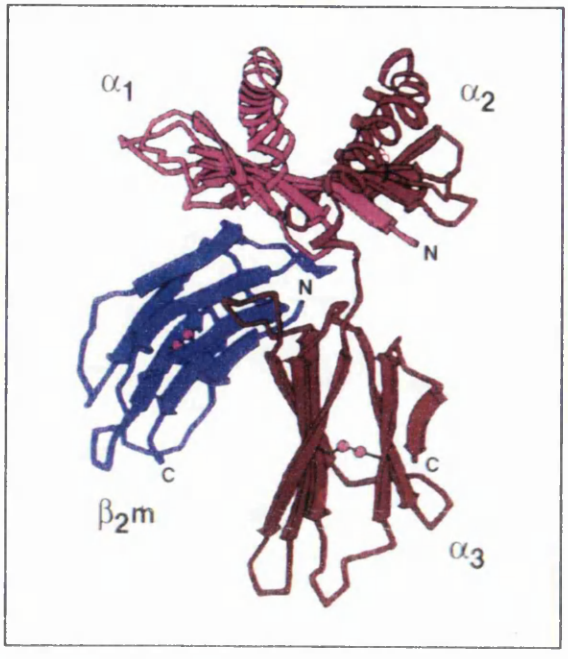
Fig.1.6 The MHC locus



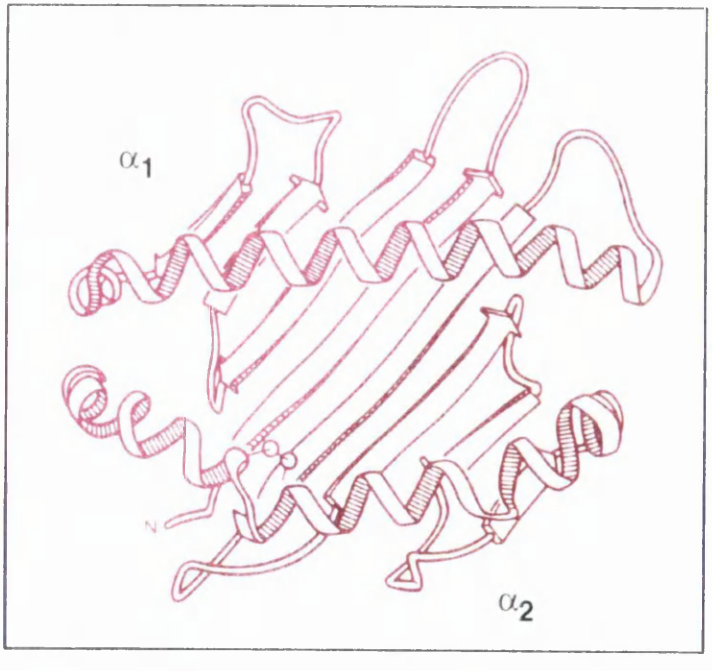
Reproduced from Immunol. Today 14 (1993)

Fig.1.7 Schematic representation of a MHC class I molecule

a) positions of heavy chain and β_2m domains



b) structure of the peptide binding groove



Reproduced from "HLA and disease" (1994) R.Lechler (ed)

1.6.1.2 Antigen processing and assembly of MHC class I molecules

The formation of class I heterodimers takes place in the endoplasmic reticulum (ER) soon after the translation of the α-chain and the β_2m molecules.

Endogenous proteins are degraded by a unique proteolytic cytoplasmic protein complex to yield random 8-, 9- and 10-mer peptides. These peptides are then transported into the ER by the transporter-associated for antigen presentation, or TAP, proteins (DeMars *et al.*, 1985; Salter & Cresswell, 1986; Trowsdale *et al.*, 1990; Spies *et al.*, 1992). Peptides from proteins which contain ER-specific targeting signals enter the ER in a TAP-independent manner, although these are a minority (Henderson *et al.*, 1992). Peptides from within the ER bind into the groove of the MHC α-chain and this facilitates complex formation with β_2m (Townsend *et al.*, 1989). The assembly process is energy dependent, requiring ATP, and it is thought that there are other as yet unidentified proteins involved at this stage (Levy *et al.*, 1991). Only at this point can the assembled molecules be transported to the cell surface via the golgi apparatus (Yewdell *et al.*, 1988; Nuchtern *et al.*, 1989; Yewdell & Bennink, 1989). The complexes are anchored into the plasma membrane by a hydrophobic tail and persist at the cell surface until they are degraded.

1.6.1.3 Functional significance of MHC class I

Presentation of endogenous peptide to the CD8+ CTL component of the immune response protects against intracellular pathogens which are unreachable by neutralising antibody. The MHC class I molecule, when complexed with peptide, can be recognised by the TCR. In the case of class I interactions, the binding of TCR to the MHC complex is stabilised by the CD8 molecule (Salter *et al.*, 1990).

The MHC class I molecule, which is present at the cell surface complexed with antigen, is exposed to a constant CTL surveillance. MHC class I alleles express different MHC molecules which possess peptide binding grooves with distinct architectures. Because of this, not all viral peptides bind into the groove of every MHC class I molecule and this leads to selective presentation of certain viral peptides by specific subclasses of MHC class I.

1.6.2 MHC class II

MHC class II molecules, encoded in humans by the HLA-DP, -DQ and -DR genes, are expressed by only a restricted set of cell types including B-lymphocytes, macrophages and monocytes, and other antigen presenting cells (APC) such as those of the reticuloendothelial system. Class II molecules present exogenous antigen to the CD4+ T-cell compartment. In this way the extracellular environment is continually sampled and offered for T-cell surveillance.

The class II molecules have a similar structure to their class I counterparts as defined by X-ray crystallography (Brown *et al.*, 1988). The Class II molecule is a dimer consisting of α- and β-chains which both have two domains. The antigen binding site is also similar to class I, being formed from the α_1 and β_1 domains of the two chains. The binding site itself is open ended and therefore can bind much larger peptides than the closed site of class I molecules.

The antigen processing pathway for MHC class II is quite distinct from that of class I. Exogenous antigen, either native or complexed with neutralising antibody, is taken into the APC via the endosomal pathway (Cresswell, 1985) and nested sets of peptides 13-25 amino acids in length are generated by cellular enzymes. The MHC class II molecules are synthesised in the ER and targeted to a distinct endosomal compartment (Guagliardi *et al.*, 1990). Irreversible complex formation occurs here between peptide and MHC molecule. The complexes are transported to the cell surface where they have a half life of 36 hours. Recognition of antigen presented on MHC II requires interaction with the TCR and CD4.

1.6.3 Immune response to a viral agent

The normal immune response to a virus consists of the production of antibodies and cytokines, and the induction of natural killer cells and T-cells. In most cases, CTLs play a critical part in the antiviral defense mechanism, identifying and eliminating virus-infected cells (Nowak & Bangham, 1996). Thus, the main protective immune response to a viral agent is generated against intracellular viral peptides presented to CTLs on MHC class I molecules at the surface of the infected cell (Townsend *et al.*, 1986; Townsend & Bodmer, 1989). The existence of memory T-cells, which are less well understood, is also critically important in the immune response to a persistent viral infection.

1.6.4 Immune evasion by viruses

For a virus to establish a persistent infection of any kind it must be able to achieve a balanced host-virus relationship. Immune evasion is the most common way to do this, and can be achieved in a variety of ways. Some of these are described below.

Antigenic variation is a feature of viruses such as influenza and HIV and results in the host immune response being constantly avoided (reviewed by Koup, 1994). In the case of HIV, viral antigens which are key targets for the host immune response are constantly mutating, and selection occurs in favour of those mutations which render the viral protein undetectable, however briefly, by the host immune system (Phillips *et al.*, 1991; Couillin *et al.*, 1994). In the case of influenza virus there is gross antigenic switching of the haemaglutinin and/or neuraminidase proteins between viral strains during coinfection, resulting in an entirely novel protein to which there is no primary or memory response in the human population (Webster *et al.*, 1982; reviewed by Kilbourne, 1987).

Persistent infection of what is termed a privileged site is another mechanism of evasion of the host immune system. Certain sites within the body are not subjected to immune surveillance, thus a virus may remain undetected in any such site indefinitely (Barker & Billingham, 1977). An example of such a site is the central nervous system, which is protected from lymphocyte infiltration by the blood/brain barrier and does not express MHC class I or class II molecules (Joly *et al.*, 1991). These features make it an excellent site for viral persistence; HSV-1 and VZV (section 1.3.1-1.3.2) both persist in a latent state in sensory ganglia.

Viruses which can adopt a latent state, in which the expression of antigenic viral proteins can be minimised or eliminated, are more likely to be able to persist in the host, undetected by CTL surveillance. HSV-1 and EBV utilise this mechanism (Stevens, 1994).

Immune modulation is another example of a mechanism by which viruses persist in the infected host. Viruses such as HCMV and adenovirus types 2 and 12 cause a decrease in cell surface MHC antigen expression (Bernards *et al.*, 1983; Anderson *et al.*, 1987; Browne *et al.*, 1990).

1.7 Immunity to EBV

Proliferation of B-cells harbouring latent virus is controlled by the cellular component of the immune response, consisting of non-HLA-restricted natural killer cells and HLA-restricted CTLs (Misko *et al.*, 1980; reviewed by Moss *et al.*, 1992). Neutralising antibody limits the spread of virus from the oropharynx and controls further spread of infectious virus following reactivation events (reviewed by Lee, 1994).

Following primary infection there is a constant level of immunity to EBV which is maintained throughout life (Rocchi *et al.*, 1973). EBV establishes lifelong infection by adopting a latent state in lymphocytes and in this way it escapes immune surveillance by expressing only a restricted subset of viral genes. The only genes expressed in the immunocompetent host in B-cells harbouring latent virus are EBNA-1 and LMP-2 as discussed in section 1.4.6 (Qu & Rowe, 1992; Tierney *et al.*, 1994; Chen *et al.*, 1995). None of the lytic genes to which antibodies are made during the initial stages of infection are expressed.

1.7.1 Humoral immune response to EBV

The humoral response to EBV peaks following primary infection when the host seroconverts. The first wave of antibodies is directed against lytic cycle antigens, mainly viral capsid antigen (VCA) and early antigen (EA) (Rosen *et al.*, 1977). Production of neutralising antibody is an important factor in controlling initial spread of the virus and thus a determinant for eventual host viral load. Longterm antibody titres to EBV VCA can be used as an indirect indicator of viral load (Henle & Henle, 1979).

The persistently infected host maintains antibody titres to EBV antigens throughout life; antibody titres are maintained by reactivation events which occur naturally in the infected host (reviewed by Lee, 1994). Reactivation results in transient expression of the immunogenic lytic phase gene products, triggering a recurrent wave of IgG production to EA, VCA and the major viral envelope glycoprotein gp350/220 (Thorley-Lawson & Geilinger, 1980, van Grunsven *et al.*, 1993). Serum IgG specific for EBNA-1 can also be detected lifelong in healthy virus carriers (Lee, 1994).

1.7.2 Cellular immune response to EBV

In an attempt to understand how the virus evades CTL recognition in the latent state, extensive studies have been performed to determine which MHC class I alleles present which viral epitopes. It has been found that peptides from all the latent proteins, with the exception of EBNA-1, are presented by at least one HLA class I allele (Khanna *et al.*, 1992; R.J.Murray *et al.*, 1992). Several epitopes with structures compatible with presentation on class I molecules have been shown to be recognised by CTLs. A selection of HLA-A and -B alleles and the epitopes of EBV latent proteins which they are known to present is shown in Table I.v.

Distinct peptides from the same EBV latent protein can be presented by multiple HLA alleles. This is due to differences in the architecture of the peptide binding grooves of different HLA alleles, demonstrated by studies using recombinant vaccinia virus expressing EBV latent genes (R.J.Murray *et al.*, 1990; 1992; Khanna *et al.*, 1991; 1992). Individual alleles may also present several epitopes from the same protein or from different proteins. For each subclass of MHC I molecule there are peptides which bind most stably into the grooves of the specific MHC molecules and therefore persist longer in the MHC complex at the cell surface, thus generating the strongest response. These are referred to as immunodominant epitopes. For example, in the case of HLA-A11 there are multiple peptides of the EBNA-3b protein presented, including a 9-mer and a 10-mer. There is a detectable CTL response to both of these peptides, however the 9-mer is immunodominant over the 10-mer. It was found that the 9-mer bound much more stably into the A11 peptide binding groove than did the larger peptide (Gavioli *et al.*, 1993; 1995).

Some examples of immunodominant epitopes and MHC I alleles are shown in Table I.vi.

Table I.v Presentation of epitopes of EBV latent proteins by HLA class I alleles

protein	*alleles presenting epitopes*
EBNA-1	none known
EBNA-2	A2, B7, B18
EBNA-3a	A2, A24, B8, B27, B40, B51
EBNA-3b	A11
EBNA-3c	A2, A11, A24, B7, B8, B27, B44
LMP-1	B24, B40, B51
LMP-2	A2

Khanna *et al.*, 1992; Moss *et al.*, 1992; R.J.Murray *et al.*, 1992; Kurilla, 1993.

Table I.vi HLA class I immunodominant EBV epitopes

HLA class I allele	*latent protein presented*	*immunodominant epitope*
A11	EBNA-3b	aa 416-424
B35	EBNA-3a	aa 458-466

De Campos-Lima *et al.*, 1993; Lee *et al.*, 1993; Lee *et al.*, 1995.

EBNA-1 has been repeatedly shown to be a poor target for the immune response (Rickinson *et al.*, 1992; Masucci & Ernberg, 1994). The lack of recognition of any peptides from the EBNA-1 protein is of obvious value to the virus as EBNA-1 must be expressed in every infected cell in order to maintain the viral episome. This is of particular importance when considering the pathogenesis of EBV-associated BL where EBNA-1 is the only viral protein expressed in the tumour cells.

It has recently been shown that the large glycine-alanine internal repeat region of the EBNA-1 protein, to which no clear functional properties have previously been attributed, is crucial to the inhibition of antigen processing and the absence of peptide epitopes of EBNA-1 from MHC class I restricted presentation (Levitskaya *et al.*, 1995). There is still little known about the possibility of a MHC class II-restricted CTL response to EBNA-1, although a recent study has identified an HLA-DR1-restricted epitope recognised by CD4+ CTLs (Khanna *et al.*, 1995). In this study, however, it was noted that these CTLs were not capable of lysing target cells endogenously expressing the antigen.

1.7.3 Immune evasion by EBV in healthy populations

The phenomenon of viral escape mutants, generated by *in vivo* selection pressures from the host immune response acting on spontaneous viral mutations, has been acknowledged for some time. As discussed in section 1.6.4, viruses such as HIV and influenza virus evolve rapidly in order to survive in the face of a specific host response. Herpesviruses in general tend not to be associated with this rapid viral evolution; there are many types of herpesvirus, which infect a huge and diverse range of creatures, and as a virus family they are well conserved. This would indicate that in most cases an evolutionary balance has been reached, and therefore that the selection pressures acting on the viruses are less strong than in the case of a relatively new host-virus relationship such as that of humans and HIV. There is, however, evidence for selection pressures in relation to EBV.

Studies have been carried out within an isolated population in Papua New Guinea which has an unusually high frequency of the HLA-A11 allele (de Campos-Lima *et al.*, 1993; 1994). On investigation of the EBV seropositive individuals in this population it was found that, in all of the six cases

studied, the immunodominant epitope presented on A11, representing aa416-424 of EBNA-3b, was mutated. The mutations were found in positions which rendered the epitope unable to bind into the A11 groove, thus abolishing presentation of the epitope and therefore the immunodominant CTL response normally seen in A11-positive individuals. It was postulated that this was a result of the selection pressure on the virus in this population, which had resulted in the virus evolving and thus evading the immunodominant immune response.

A second study was carried out looking at populations in parts of Africa which had high frequencies of the B35.01 allele (Lee *et al.*, 1995). The immunodominant response in B35.01-positive individuals occurs against the epitope of EBNA-3a representing aa458-466. When the epitope was investigated in this cohort it was found to be mutated in approximately 50% of Gambian and Kenyan isolates. Of the three types of mutations observed, one had no effect on CTL recognition while the other two gave 100-1000 fold weaker recognition. None of the observed mutations totally abolished the CTL response to this epitope.

In summary, in Papua New Guinea where A11 has an allele frequency of up to 50%, the target epitope was mutated in 100% of cases studied and this mutation abolished CTL recognition of the peptide. In the African cohort, where B35.01 has an allele frequency of 30%, the target epitope was mutated in only 50% of cases and mutations did not completely abolish CTL recognition. These results may reflect the effect of different selection pressures acting on the virus within different populations. The selection pressure driving the occurrence of escape mutants in target epitopes may be greater in a population where the frequency of the presenting allele is higher. Another factor influencing the selection pressure may be the strength of the immune response to the epitope presented by the allele in question. In support of the latter possibility, it is known that the CTL response to aa416-424 of EBNA-3b presented by A11 is a stronger response than that generated by aa458-466 of EBNA-3a presented by B35.01.

1.7.4 Immune response to EBV in disease

1.7.4.1 Immune response to EBV in Burkitt's lymphoma

BL cells in culture, immediately following their isolation from fresh tumour material, display a Lat I pattern of EBV latent gene expression (section 1.4.6). Additionally, there is no detectable expression of adhesion molecules such as LFA-3 and ICAM-1. If cultured continually, over time some BL-derived cell lines will drift through intermediate expression patterns, e.g. Lat II, and eventually acquire a Lat III phenotype, with full expression of viral latent proteins and markers of cellular activation (Rooney *et al.*, 1986; Rowe *et al.*, 1987; 1992). This suggests that the primary BL cells have the potential for Lat III expression, and that the absence of expression by BL cells *in vivo* is due to an intact host immune response to EBV. However, EBV-associated BL occurs in geographical locales where malaria is holoendemic (Epstein & Achong, 1979). It has been shown that during certain types of malaria, T-cell control of latent EBV infection is diminished or lost (Moss *et al.*, 1983; Whittle *et al.*, 1984). This implies that there could be a role for transient immune suppression in the early stages of tumour development.

One of the features of the pathogenesis of BL is the ability of EBV-infected B-cells to evade detection by the immune response after acquisition of the malignant phenotype (section 1.4.7.2). Several mechanisms have been suggested for this evasion, all of which may play some contributory role to the overall pathogenesis of the disease. The ability of the EBV-positive tumour cells to express only EBNA-1 is thought to play a large part in the survival of these cells. As discussed in section 1.7.2, EBNA-1 is not presented by MHC class I molecules at the cell surface and can thus not be detected by host CTL surveillance.

Another such mechanism which has been demonstrated involves downregulation of MHC class I expression at the surface of infected cells. Selective downregulation of HLA class I alleles occurs in various types of tumour, driven by the immunoselective process *in vivo* (Pandolfi *et al.*, 1991; Moller & Hammerling, 1992; Browning *et al.*, 1993). This has been demonstrated in the case of BL for the HLA-A11 allele (Andersson *et al.*, 1991; Gavioli *et al.*, 1992). The mechanism by which this downregulation occurs is thought to involve lack of cellular transcription factors and the occurrence of genetic abnormalities (Imreh *et al.*, 1995); the role played by EBV in these events is unclear.

A second mechanism of immune evasion which has been reported is the induction of high levels of expression of IL-10 in BL cell lines by EBV (Magrath, 1990; Matsuda *et al.*, 1994). IL-10 has been shown to have a wide variety of stimulatory effects on B-cells. Of greater relevance in the context of BL, it has been found to suppress the function of helper T-cells (Fiorentino *et al.*, 1989) and down-regulate HLA class I expression (Matsuda *et al.*, 1994). EBV is known to encode a homologue of IL-10 (v-IL-10) which shares at least some of the biological properties of the human form (Moore *et al.*, 1990). Unlike its cellular counterpart, it does not enhance MHC II expression, however it does inhibit IFN-γ production by helper T-cells. This indirectly inhibits the induction of MHC antigen production. Expression of v-IL-10 may occur at some stage in the development of BL.

Downregulation of the cell surface adhesion molecules LFA-3 and ICAM-1 has also been demonstrated in primary BL cells *in vitro* (Gregory *et al.*, 1988). This feature of BL cells could inhibit their detection by host CTLs due to the lack of necessary cellular interactions. It is not known whether EBV contributes to this effect; it has been shown that expression of LMP-1 induces these molecules and LMP-1 is not expressed in BL tumour cells.

1.7.4.2 Immune response to EBV in nasopharyngeal carcinoma

Tumour cells of NPC contain clonal EBV genomes and express a Lat II phenotype, which includes expression of EBNA-1 and LMP-1 and -2 (Brooks *et al.*, 1992). Although much is known about the pathogenesis of EBV in B-cell disease, it has been more difficult to study epithelial tumours such as NPC, as the tumour cells do not grow out *in vitro* and only rarely has there been successful transplantation into nude mice (Busson *et al.*, 1988; Li *et al.*, 1992).

The EBV LMPs are potentially good targets for the cellular immune response. Their expression on NPC cells suggests that there is no recognition of these antigens by CTLs *in vivo* in NPC patients. It has been demonstrated that NPC patients do mount cell-mediated immune responses to EBV antigens *in vitro*, although these may be slightly weaker than those of healthy controls (Chan *et al.*, 1979).

One of the striking features of NPC is the occurrence of unusually high antibody titres to EBV VCA and EA (Henle & Henle, 1976). In particular, raised IgA titres to these antigens are unique to NPC and precede the onset of disease by several years, suggesting that reactivation of EBV to productive replication occurs before development of the tumour.

1.7.4.3 Immune response to EBV in Hodgkin's disease

As discussed in section 1.7.1, there are raised antibody titres to EBV in most cases of HD, both EBV-associated and non-EBV-associated. There are also raised titres to other herpesviruses e.g. HHV-6 (section 1.5.2). One possibility was that this merely reflected the immunosuppressive nature of HD, which was enabling increased viral reactivation and replication to occur. The fact that the raised titres to EBV and HHV-6 do not occur in the same individuals (Clark *et al.*, manuscript in preparation) suggests that there is not just a general increase in herpesvirus load. There is not a clear relationship between EBV status of the tumour and serological titres. However, recent findings from our group suggest that EBV-associated cases as a group have higher VCA titres compared to non-EBV-associated cases.

There is a threefold higher risk of developing HD in people with a history of IM (Munoz *et al.*, 1978). It is possible that an increased viral load results either as a consequence of a high infectious dose at primary infection, or as a result of a poor immune response to the virus. This would lead to an increased likelihood of reactivation events occurring and therefore increase the chance of an RS cell precursor becoming infected with the virus. It must be emphasised that there is no direct evidence available to date that past history of IM predisposes to HD which is EBV-associated.

There is evidence to suggest that cellular immune defects precede the development of HD (Del Giacco *et al.*, 1985; Merk *et al.*, 1990). We investigated this hypothesis by studying the overall cell-mediated immune response to EBV in HD. These experiments are described in chapter three of this thesis.

RS cells in HD have a Lat II pattern of EBV gene expression, comprising expression of EBNA-1 and the LMPs (Pallesen *et al.*, 1991; Deacon *et al.*, 1993; Grasser *et al.*, 1994), as in NPC (section 1.7.4.2). A genetic co-factor has been described in relation to NPC (De The, 1982) and it may be that this is also true for HD. The HLA genes are a good starting point for the investigation of this hypothesis as they have a major influence on immune handling of infectious diseases and neoplastic outgrowths *in vivo*. Experiments carried out to assess the involvement of HLA class I in HD are described in chapter four of this thesis.

Chapter 2

General Materials & Methods

2.1 CELL BIOLOGY

The following sections outline materials and methods frequently employed during the cell biology aspect of these studies. Sources of commonly used reagents and consumables are listed in Table II.i and routinely used culture media are listed in Table II.ii. Origins of cell lines used throughout the work of this thesis are detailed in Table II.iii; sources of these cell lines are listed in Appendix A. Supplier details are given in Appendix B.

All clinical samples, cell lines, peripheral blood mononuclear cells (PBMCs) and viral preparations were handled in a Class II microbiological safety cabinet. All cell cultures were maintained at 37°C with 5% CO_2 during these studies unless otherwise stated. During routine procedures it was often necessary to change the density at which cells were resuspended; this was achieved by centrifugation followed by resuspension at an appropriate density. Cells were centrifuged at 400*g* for ten minutes unless otherwise stated.

2.1.1 Collection of blood samples and separation of PBMCs

2.1.1.1 Collection of blood samples

Samples of peripheral and umbilical cord blood were received diluted 1:2 in travel medium (Table II.ii).

2.1.1.2 Separation of PBMCs from whole blood using a Ficoll-paque gradient

Peripheral blood samples were diluted a further 1:2 in wash medium (Table II.ii), containing preservative-free heparin at 30U/ml, before being layered onto a Ficoll-paque gradient at room temperature. In order to obtain the necessary *g*-force at the interface, 30ml of diluted blood were layered onto 15ml of Ficoll-paque in a 50ml centrifuge tube and the gradient centrifuged at 1500*g* for 20 minutes at room temperature. The interface band was removed using a Pastette® (Alpha Labs) and the cells washed twice in wash medium to remove any remaining Ficoll. PBMCs obtained in this way were counted as described in section 2.1.1.4.

Table II.i Sources of commonly used materials

material	*source*
RPMI 1640 medium	Life Technologies
Hank's balanced salt solution	Life Technologies
Foetal bovine serum	Life Technologies
Penicillin/streptomycin	Life Technologies
Gentamicin	Life Technologies
Fungizone®	Life Technologies
Hepes buffer	Life Technologies
Tissue culture grade distilled water	Life Technologies
Ficoll-paque	Pharmacia Biotech
Cyclosporin A	Sandoz Pharmaceuticals
Preservative-free heparin	Leo Laboratories
DMSO	Sigma Chemical Company
TPA	Sigma Chemical Company
Other chemicals	Sigma Chemical Company

Table II.i Sources of commonly used materials (continued)

material	*source*
Plasticware	
tissue culture flasks, 50cm^2 or 250cm^2	Costar (UK) Ltd.
tissue culture flasks, 750cm^2	Nunclon
96-well microtitre plates	Nunc
sterile pipettes, 5ml, 10ml or 25ml	Costar (UK) Ltd.
polypropylene tubes, 15ml or 50ml	Greiner Labortechnik
cryostorage tubes, 1.8ml	Nunc
ultra-clear ultracentrifuge tubes, 35ml	Beckman Instruments
12 x 75mm sterile tubes	Coulter Electronics Ltd.
screw-cap microfuge tubes	Alpha Laboratories Ltd.
Pastettes®	Alpha Laboratories Ltd.
Rainin pipette tips	Anachem

Table II.ii Media and reagents used in cell culture

solution	*constituents*	*concentration*
complete medium	RPMI 1640 medium	
	foetal bovine serum	10%
	L-glutamine	2mM
	penicillin/streptomycin	400IU/ml; 400mg/ml
travel medium	RPMI 1640 medium	
	foetal bovine serum	20%
	L-glutamine	2mM
	penicillin/streptomycin	400IU/ml; 400mg/ml
	gentamicin	150μg/ml
	fungizone	2.5MCG
	Hepes buffer	25mM
	preservative-free heparin	30U/ml
wash medium	Hank's balanced salt solution	
	foetal bovine serum	2%
	penicillin/streptomycin	400IU/ml; 400mg/ml
freezing medium	foetal bovine serum	92%
	DMSO	8%
cyclosporin A	stock solution, dissolved in analytical grade absolute alcohol	1mg/ml
	working solution, diluted in complete medium	5μg/ml
TPA	stock solution, dissolved in DMSO	200μg/ml

Table II.iii Commonly used cell lines

cell line	*description*
B95-8	EBV infected marmoset B-cell line
HB82	Mouse hybridoma line secreting BB7.2 antibody
Jijoye	EBV-infected human Burkitt's lymphoma-derived B-cell line
J-Jhan	T-cell line derived from acute T-cell leukaemia (clone of Jurkat)
Raji	Human Burkitt's lymphoma line - infected with defective EBV
VES	EBV-infected B-lymphoblastoid cell line

2.1.1.3 Separation of umbilical cord blood mononuclear cells (CBMCs) using a Ficoll-paque gradient

The umbilical cord blood samples used for these studies were diluted 1:3 in wash medium containing preservative-free heparin at 30U/ml before being layered onto a Ficoll-paque gradient. The subsequent procedure was as outlined in section 2.1.1.2 for the separation of PBMCs.

2.1.1.4 Cell counting

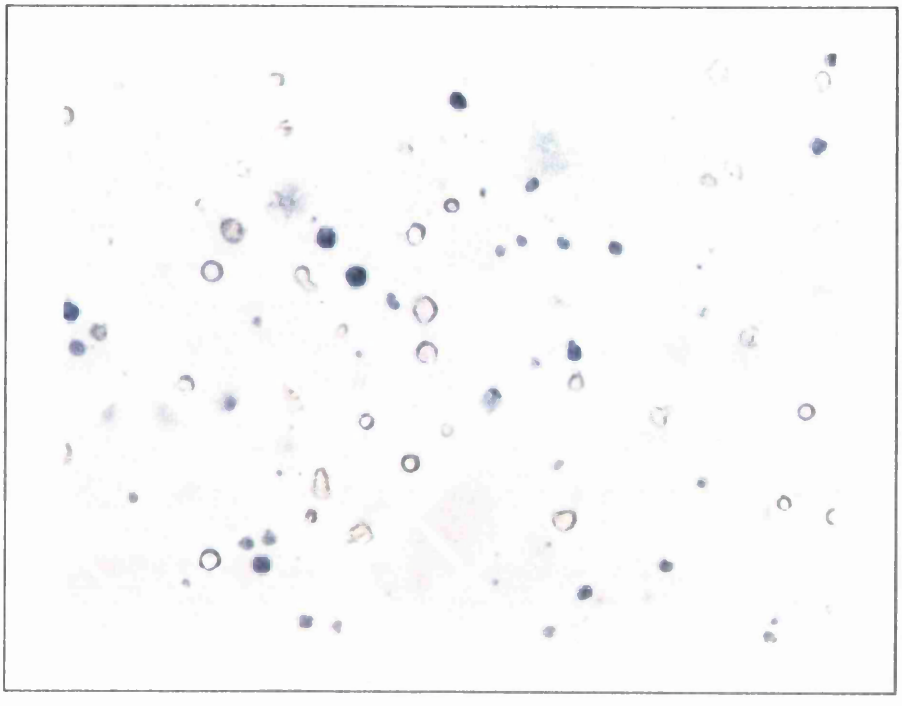
PBMCs and CBMCs were fixed in two volumes of 2% acetic acid with a trace of crystal violet. This fixative was chosen because it causes lysis of any contaminating erythrocytes and so a true mononuclear cell count is obtained. The cells were fixed for five minutes before being counted in a Neubauer haemocytometer.

Following defrosting cells from liquid nitrogen, or for the purpose of counting cell lines in culture, trypan blue staining was used to estimate percentage viability. The cell suspensions were stained for five minutes in a 0.2% solution of trypan blue in saline (Sigma Chemical Company) before being counted as described above. Non-viable cells take up the stain due to altered permeability of the cell membrane, thus appearing blue when examined (Fig.2.1).

2.1.1.5 Freezing of cells

Cell pellets were chilled to 4°C and resuspended in 1ml aliquots containing up to $1x10^7$ cells in freezing medium (Table II.ii). The aliquots were placed in a Nalgene Cryo 1°C freezing container® at -80°C overnight. The containers were filled with isopropyl alcohol which allows the cells to cool slowly (approximately 1°C/minute) thus ensuring a good percentage recovery on defrosting. Following freezing, cells were stored in liquid nitrogen.

Fig.2.1 PBMCs stained with trypan blue



Cells are pictured against the graticule of a haemocytometer. Viable cells appear bright and refractile, whereas dead cells which have taken up the stain appear dark blue (x400).

2.1.2 Preparation of EBV viral stocks and infection of PBMCs

2.1.2.1 Preparation of concentrated EBV stocks

The B95-8 cell line, which is productively infected by EBV, was cultured in complete medium at 35°C and expanded by splitting 1:2 twice weekly with total medium replacement. When the culture reached 240ml in a 750cm^3 flask the medium was replaced and the culture left for four days without further attention.

The culture supernatant containing the virus particles was then clarified by centrifugation at 500*g* for ten minutes to remove cells. The supernatant was filtered using a 0.45μm bottle filter (Costar UK Ltd.), on ice, and ultracentrifuged at 21,500rpm for 2.5 hours in a prechilled SW28 Beckman rotor at 4°C. The supernatant was discarded and the virus pellets placed on ice and resuspended in a total volume of 1.5ml cold complete medium for one hour, to allow complete resuspension of the virus particles to take place. The viral suspension was aliquotted into 6 x 250μl aliquots in screw-capped microfuge tubes and the aliquots stored at -80°C until use.

2.1.2.2 Titration of EBV stocks on CBMCs

For this assay 8x10^6 CBMCs, recovered from a Ficoll-paque gradient, were split into eight sterile 12cm x 75mm tissue culture tubes (Table II.i) and the cells pelleted. Viral stock was thawed at 37°C and ten-fold serial dilutions performed, on ice, from neat virus down to a 10^{-6} dilution. Seven of the cell pellets were resuspended in each of the serial viral dilutions and the remaining pellet resuspended in complete medium for use as an uninfected control. The tubes were incubated at 37°C with 5% CO_2 for one hour. After incubation, 2ml of wash medium were added to each tube and the cells pelleted. Cell pellets were resuspended in 1ml of complete medium containing 20% FBS and plated into a 96-well microtitre plate as four replicate wells of 200μl. Cultures were fed weekly by 50% medium replacement, and transformation was assessed (Fig.2.2) after 3-4 weeks.

Transformation appears as numerous refractile clumps of cells, with cytoplasmic projections visible on the outer cells of each clump (Fig.2.2). Transformed cells display the characteristic "hand-mirror" shape associated with EBV-infected lymphoblastoid cells and this is most easily visualised in the smaller clumps.

The result of the titration was scored as follows: the dilution at which transformation was evident in 50% of the wells was taken as the inverse of the viral titre.

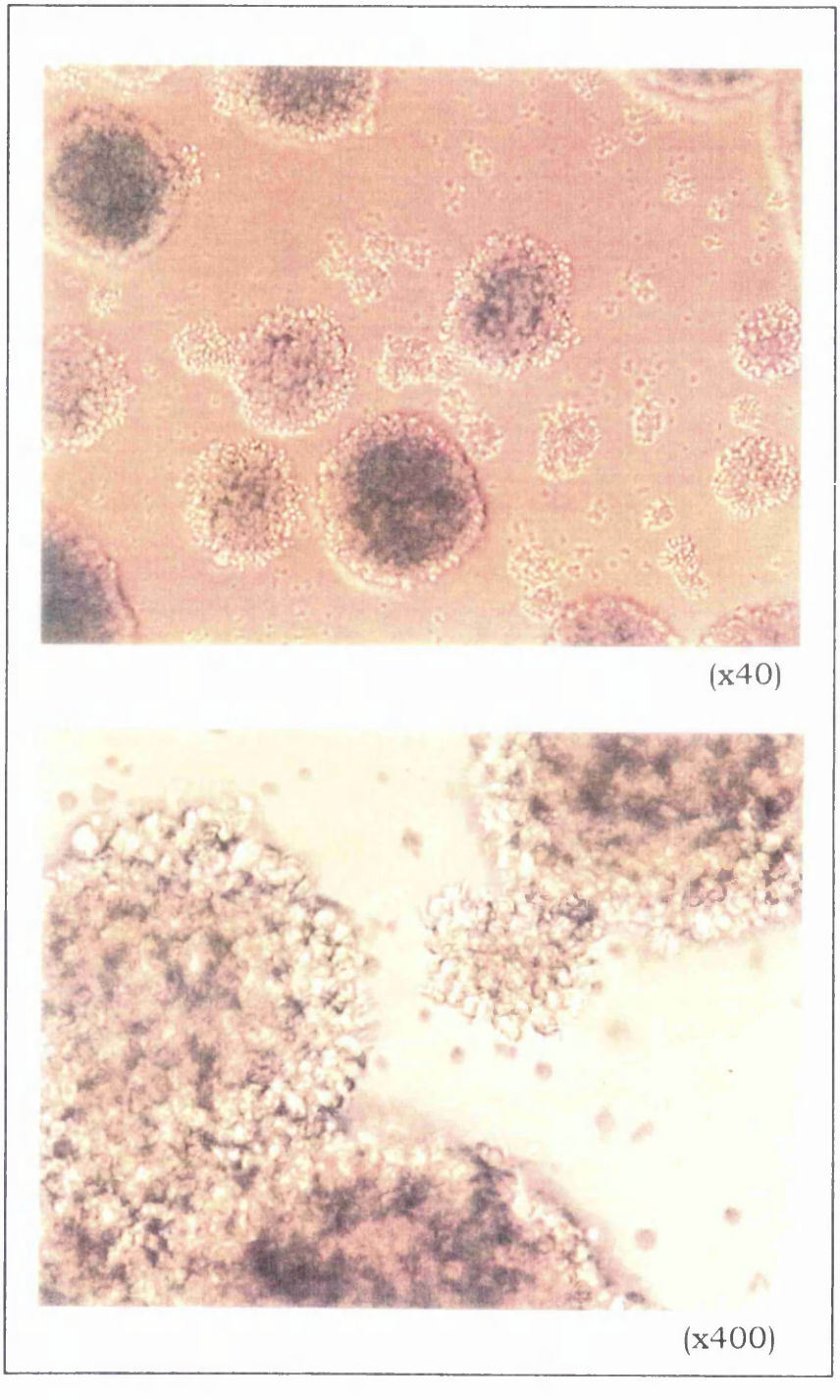
2.1.2.3 EBV infection of PBMCs

PBMCs were counted and pelleted in a sterile 75mm x 12mm tissue culture tube (Table II.i). The cell pellet, containing a minimum of $2x10^5$ cells, was resuspended in 250µl EBV stock (thawed at 37°C) and the volume made up to 500µl with complete medium. The cell suspension was incubated for one hour at 37°C with 5% CO_2.

After this time the cells were washed and resuspended at a density of $3x10^6$ cells/ml in complete medium containing cyclosporin A (CsA) at a concentration of 0.1µg/ml. The cells were plated into 96-well microtitre plates in 200µl/well at a density of $3x10^6$ cells/ml, and the surrounding wells filled with 200µl sterile distilled water to maintain humidity across the plate. The plate was incubated at 37°C with 5% CO_2 and fed weekly by 50% medium replacement.

Evidence of transformation could usually be seen 1-2 weeks following infection, as described above (section 2.1.2.2) for CBMCs. In some cases transformation was not evident until 6-8 weeks after infection, and in these circumstances it was assumed that the B95-8 virus infection had failed to transform the cells and a spontaneous transformation event, involving latent virus present in the PBMCs, had taken place.

Fig.2.2 Appearance of EBV-transformed B-cells *in vitro*



(x40)

(x400)

2.1.3 Miscellaneous cell biology procedures

2.1.3.1 Cytospins

Cells were pelleted at 400*g* for five minutes and resuspended at a density of $1x10^6$ cells/ml; 100µl of this suspension were used for each cytospin, resulting in $1x10^5$ cells per spot. Cytospins were centrifuged at 400*g* for ten minutes in a Shandon Cytospin II using APES-coated slides (section 2.2.7.1). Cytospins were air dried and fixed in either ice-cold acetone or methanol for 20 minutes and stored at -20°C for up to 8 weeks.

2.1.3.2 Preparation of cell lysates for PCR

Cell pellets containing $2x10^5$ cells were defrosted from storage at -80°C. Pellets were resuspended in 50µl non-ionic detergent lysis buffer (Table II.v) containing proteinase K (Sigma Chemical Company) at a concentration of 60µg/ml. The cell suspensions were incubated at 56°C for one hour to lyse the cells and allow digestion of proteins and then steamed at 95°C for ten minutes to inactivate the protease.

2.1.3.3 Mycoplasma screening

MRC-5 cells were seeded into slide flasks (Nunclon) and cultured in complete medium at 37°C with 5% CO_2 until the cells were just sub-confluent. At this time 500µl of supernatant from the test culture were added to the slide flask and incubated for three days. A negative control consisting of culture medium only was included in each assay. Following incubation, the slides containing the monolayers were snapped off the flasks and the cells fixed in a 3:1 solution of methanol and glacial acetic acid. Cells were fixed for ten minutes and immersed for a further ten minutes in a 1:20,000 dilution of Hoescht 33258 stain in HBSS without phenol red. Slides were washed for 2x five minutes in distilled water. Slides were mounted while wet.

Slides were viewed using a UV light microscope. The presence of mycoplasma is seen as strings of fluorescent dots distributed around the nucleus in a uniform manner. It is essential to bear in mind the EBV status of the samples to be screened as EBV can mimic the effect of mycoplasma using this technique.

2.2 MOLECULAR BIOLOGY

This section details routinely used materials and methods involved in the molecular biological investigations carried out for this thesis. Commonly used equipment, consumables and chemicals are listed in Table II.iv and stock solutions and buffers are detailed in Table II.v. All chemicals used were of analytical quality. Deionised water from a MilliQ water filtration system (Millipore UK Ltd.) was used in all procedures with the exception of PCR and resuspension of high molecular weight DNA. For these manipulations either sterile ultra-pure deionised water, obtained from a reverse osmosis filtration system (Millipore UK Ltd.), or sterile nuclease-free water supplied by Pharmacia Biotech were used. A list of suppliers is detailed in Appendix B.

2.2.1 Extraction and manipulation of high molecular weight DNA

2.2.1.1 Extraction of DNA from cell pellets

Cell pellets were digested in TNE buffer containing 0.5% sodium dodecyl sulphate (SDS) and Proteinase K at a concentration of 0.1mg/ml. Five millilitres of buffer were used for a pellet of $1x10^7$ cells; the suspensions were incubated at 37°C overnight with continual shaking. The resulting lysates were allowed to cool to room temperature before performing organic solvent extractions for removal of protein. All subsequent steps were performed at room temperature unless otherwise stated.

An equal volume of phenol equilibrated with Tris pH 8.0 (Rathburn Chemicals Ltd.) was added to the lysate in a 15ml polypropylene tube and mixed thoroughly, but gently, by inversion. The mixture was centrifuged at 3000rpm for ten minutes in a benchtop Beckman GPR centrifuge to separate the aqueous and organic phases. The aqueous phase was removed and transferred to a fresh tube. This step was carried out using a wide bore Pastette® (Alpha Laboratories Ltd.) to prevent shearing of the high molecular weight (HMW) DNA.

Table II.iv Sources of routinely used equipment, consumables and chemicals

material/item	*source*
Apparatus for PAGE	Bio-Rad Laboratories
Apparatus for agarose gel electrophoresis	BRL (Life technologies)
Plasticware	
Falcon tubes 15ml or 50ml	Greiner Labortechnik
screw cap microfuge tubes	Alpha Laboratories Ltd.
Petri dishes	Fisons Ltd.
Falcon 2059 tubes	A. & J. Beveridge Ltd.
pipette tips	Costar UK Ltd./Anachem
Polaroid Film type 57	Genetic Research Instrumentation Ltd.
Molecular biology grade chemicals	Sigma Chemical Company/BDH (Merck Ltd.)
Restriction enzymes	Life Technologies
Easigel 37.5:1 acrylamide mix	Scotlab
Sterile nuclease-free water	Pharmacia Biotech

Table II.v Stock solutions and buffers

solution	*constituents*
TBE buffer	90mM Tris 90mM boric acid 2.25mM EDTA adjusted to pH 8.0
TAE buffer	40mM Tris 20mM sodium acetate 20mM sodium chloride 2mM EDTA adjusted to pH 8.0
TBS buffer	0.15M sodium chloride 0.05M Tris adjusted to pH 7.6
TNE buffer	0.1M sodium chloride 10mM Tris 1mM EDTA adjusted to pH 8.0
Alkali buffer	0.5M sodium hydroxide 1.5M sodium chloride adjusted to pH 12.0
Neutralising buffer	0.5M Tris pH 8.0 3M sodium chloride 3.3% concentrated hydrochloric acid
30% acrylamide stock	29% acrylamide 1% N,N-methylene bisacrylamide
10x gel loading buffer	0.42% bromophenol blue 0.42% xylene cyanol 50% glycerol

Table II.v Stock solutions and buffers (continued)

solution	*constituents*
SSC (1x)	0.15M sodium chloride 0.015M sodium citrate
Denhardt's solution (100x)	2% Ficoll 2% BSA (fraction V) 2% polyvinylpyrolidine
Hybridisation buffer	50mM Tris pH 7.4 10mM EDTA 6x SSC 5x Denhardt's solution 10% dextran sulphate 0.1% SDS
1x kinase buffer	50mM Tris pH 7.5 1mM EDTA pH 8.0 10mM magnesium chloride 5mM DTT 1mM spermidine
1x PCR buffer	10mM Tris pH 8.2 50mM potassium chloride 0.01% gelatine
Non-ionic lysis buffer	1x PCR buffer 0.45% Tween20 0.45% NP40
AP substrate	0.1M Tris pH 8.2 200μg/ml naphthol-AS B1 phosphate 0.5% DMF 5mM levamisole

The phenol extraction was repeated twice and then a final extraction performed using chloroform as the organic solvent. Following the chloroform extraction and subsequent centrifugation, the aqueous phase was pipetted into 15ml of 98% ethanol in a 50ml centrifuge tube in order to recover the HMW DNA. The solution was swirled gently until the DNA precipitate became visible; this was then spooled out using a sealed Pastette® (Alpha Laboratories Ltd.). The pellet was washed in 70% ethanol and air dried before being dissolved in sterile nuclease-free water.

2.2.1.2 Quantitation of HMW DNA and oligonucleotides

The amount of nucleic acid present in a solution was determined using a spectrophotometer. An optical density (OD) reading at a wavelength of 260nm was used to calculate the concentration of the DNA solution, based on the fact that 50μg/ml of double-stranded DNA has an OD reading of 1.0 at a wavelength of 260nm (Sambrook *et al.*, 1989). For oligonucleotides, a 33μg/ml solution gives the same reading at 260nm. The purity of the DNA solution was determined by calculating the ratio of the OD reading at 260nm to that at 280nm. For a pure solution of double stranded DNA a ratio of 1.6 - 1.8 should be obtained. If the ratio is less than this value it suggests that there is contamination of the DNA solution with protein, or there was insufficient removal of organic solvent following extraction.

2.2.2 Polymerase Chain Reaction

Amplification of a target DNA sequence using specific primer sets was achieved by use of the polymerase chain reaction (PCR), essentially as described by Saiki *et al.* (1985).

2.2.2.1 Standard PCR reaction conditions

Standard PCR reactions were performed in a final reaction volume of 50μl. Present within each reaction were the following constituents:

DNA at 660ng/reaction (equivalent of $1x10^5$ cells)
Deoxynucleoside triphosphates (dATP, dCTP, dGTP, dTTP/dUTP) at 200μM
1x PCR buffer (Table II.v)
Amplitaq® DNA polymerase, one unit
Magnesium chloride at previously optimised concentration (section 2.2.2.2)
Primers at previously optimised concentration (section 2.2.2.2).

All reaction components were supplied by Applied Biosystems Ltd. and primers were synthesised by Cruachem.

Some PCR reactions were carried out using cell lysates which had been digested in buffer containing non-ionic detergent and protease (section 2.1.3.2). In this situation, $1x10^5$ cell equivalents were added to the reaction in a 25μl volume.

2.2.2.2 Optimisation of PCR reactions

Primers were quantified using a spectrophotometer as described for oligonucleotides in section 2.2.1.2, and the optimum concentration for amplification was titrated in a standard PCR reaction as follows. Serial two-fold dilutions of primer were performed to yield a range of final concentrations from 4μM - 0.25μM. Working primer concentration was chosen for optimal sensitivity and specificity.

Magnesium chloride concentration was also optimised for certain PCR reactions. Magnesium chloride was included in standard PCR reactions at final concentrations of 0.5mM - 2.5mM. As for primer concentration, optimal magnesium concentration was chosen as giving the most sensitive and specific result.

In order to avoid any cross contamination of PCR reactions, a designated "PCR-clean" area was used for aliquotting reagents and setting up reactions. A gown, gloves, mob cap and face mask were worn and a separate set of PCR-clean pipettes was used with plugged or positive displacement tips. Samples and negative controls were handled separately from positive controls. Oligonucleotides for other applications were synthesised within the department, whereas PCR primers were synthesised externally to ensure that they were "PCR-clean". Substitution of dUTP for dTTP in the PCR reaction allowed for use of the enzyme uracil DNA glycosylase where contamination from PCR product was suspected. It was not necessary to use this measure for any of the PCR reactions detailed in the work of this thesis.

2.2.3 Electrophoretic analysis of PCR products

2.2.3.1 Polyacrylamide gel electrophoresis for size-separation of small DNA molecules

Polyacrylamide gel electrophoresis (PAGE) was used in the separation and purification of DNA fragments of less than 0.5kb e.g. PCR products. Acrylamide solutions were made up from stock solutions (Table II.v) to a final concentration of 8% in TBE buffer and polymerised by the addition of 0.66% (w/v) ammonium persulphate and 0.12% TEMED. The gels were poured for use with the Mini-Protean II electrophoresis system (Bio-Rad Laboratories). DNA samples diluted 5:1 in loading buffer were loaded onto the gel and electrophoresed by applying 120V for 1-2 hours using TBE as the running buffer. Four hundred nanograms of the DNA fragment size marker ΦX 174 RF DNA digested with the restriction enzyme *Hae*III (Life Technologies) were run alongside each batch of DNA samples on the same gel.

Polyacrylamide gels were stained in a solution of ethidium bromide at a concentration of 0.5μg/ml in TBE for ten minutes, and then destained for a further ten minutes in deionised water before being visualised using a UV trans-illuminator.

2.2.3.2 Agarose gel electrophoresis

Different weights of agarose were dissolved in TBE buffer, by boiling the solution, to give gels of various percentages of agarose depending on the application. The agarose solution was cooled and the gels poured and allowed to solidify at room temperature before being chilled to 4°C for 30 minutes prior to use.

2.2.3.3 Purification of PCR products from agarose gels

A preparative gel of 2% Nusieve agarose (Flowgen Instruments Ltd.) containing ethidium bromide at a concentration of 0.5μg/ml was used to electrophorese the desired fragment. The gel was prepared and run using TAE as buffer, as fragments prepared using TAE performed better in subsequent enzymatic reactions than those prepared using TBE. The gel was visualised under long-wavelength UV light and the band containing the fragment of interest cut out of the gel using a scalpel. The excised piece of gel was incubated in 0.5M NaCl, 50mM MOPS pH 7.0 for 20 minutes at 70°C to melt the agarose and release the DNA into solution.

Following incubation the DNA was further purified by passage through an equilibrated Qiagen T20 tip, washing and eluting the fragment as per the manufacturers instructions. DNA was recovered by ethanol precipitation.

2.2.4 Electroblotting and hybridisation using radioactively-labelled probes

The solutions used in these procedures are specified in Table II.v.

2.2.4.1 Electroblotting of PCR products

Electroblotting was used to transfer PCR products from polyacrylamide gels onto nylon membrane for further analysis. Polyacrylamide gels were electrophoresed as described in section 2.2.3.1 and then the gels placed in alkali buffer for eight minutes to denature the DNA. Gels were placed in neutralising buffer for eight minutes and briefly equilibrated in 1x TAE transfer buffer prior to blotting. Nylon membranes (Hybond-N, Amersham International PLC) were soaked in transfer buffer and placed onto the gels

in the blotting cassettes. The cassettes were placed in the Mini-Protean II electroblotting system (Bio-Rad Laboratories) and blotting was achieved by applying 15V across the apparatus for one hour. Following electroblotting, the DNA was irreversibly bound to the membrane by crosslinking with a UV light source at 120j/cm^2 for 0.8 minutes (UV Stratalinker 1800, Stratagene). Blots were stored dry until required.

2.2.4.2 Five prime end-labelling of oligonucleotide probes

Oligonucleotides for use as probes were synthesised within the department onto commercially available columns (Applied Biosystems Ltd.). The oligonucleotides were recovered from the column by elution in 2ml aqueous ammonia solution. The oligonucleotide solutions were incubated at 55°C overnight to remove the ammonium groups, dried down in a Speed-Vac SC100 (Savant Instruments Inc.), and resuspended in 100µl sterile nuclease-free water.

Five prime end-labelling was performed in a 100µl reaction volume containing 70pmoles of oligonucleotide and 70pmoles of ^{32}P-γATP (300 Ci/mM) (ICN Biomedicals Ltd.). Twenty units of T4 polynucleotide kinase were used to catalyse the end-labelling reaction in the presence of 1x kinase buffer (Table II.v) and the reaction was incubated at 37°C for one hour. After incubation, unincorporated nucleotides were removed by passage down a Sephadex G50 gel filtration column. This was achieved using commercially available NICK columns (Pharmacia Biotech) and following the manufacturers instructions.

2.2.4.3 Hybridisation with oligonucleotide probes

PCR products were transferred from polyacrylamide gels onto nylon membrane as described in section 2.2.4.1. The specificity of the PCR products was determined by hybridisation to radioactively-labelled oligonucleotide probes.

DNA blots (section 2.2.4.1) were soaked briefly in a solution of 3x SSC, 0.1% SDS prior to the hybridisation procedure. Membranes were placed into 50ml centrifuge tubes with the bound DNA facing inwards. Pre-incubation with 2.5ml hybridisation buffer (Table II.v) was carried out for 1-2 hours in the presence of 250mg/ml Gene-bloc (I.G.I. Ltd.) to block any

non-specific binding of the probe. Incubation was carried out at 37°C on a roller mixer. After the prehybridisation step, $1x10^6$ cpm/ml of probe were added to the pre-hybridisation buffer, and the reaction incubated overnight at 37°C on a roller mixer. Formamide was added to the hybridisation buffer at a percentage which ensured hybridisation was carried out at 5°C below the Tm for the oligonucleotide probe used, in accordance with the recommendations made by Sambrook *et al.* (1989).

Following hybridisation the membranes were briefly washed twice at room temperature in a solution of 6x SSC, 0.1% SDS to remove excess probe. The blots were then subjected to two washes, for 30 minutes each wash, in the same composition buffer equilibrated to 60°C. Following washing, membranes were dried briefly and wrapped in Saran wrap (Scotlab) before being exposed to autoradiographic film (Hyperfilm, Amersham International PLC) at -80°C using an intensifying screen. Exposure times varied according to the individual experiment.

2.2.5 Cloning and sequencing of PCR products

Compositions of bacterial culture media used are given in Table II.vi.

2.2.5.1 Cloning of PCR products

In situations where PCR products were to be subsequently cloned, PCR reactions were performed in the presence of a dNTP mix containing dTTP in preference to dUTP (see section 2.2.2.1 for standard PCR reaction conditions). It had been shown previously that PCR products containing dUTPs could not be cloned efficiently (A.Philbey, personal communication). In cases where nucleotide sequencing of cloned PCR products was to be performed, the use of UlTma™ DNA polymerase (Applied Biosystems Ltd.) was preferred over the standard Amplitaq®. This was to utilise the 5' proof-reading activity of the UlTma™ enzyme which allows more faithful amplification of the target sequence.

Cloning of PCR fragments was achieved using the commercially available pCR-script™ cloning kit (Stratagene). This cloning protocol utilises a blunt end cloning technique, suitable for these PCR products, as the UlTma™ DNA polymerase generates fragments with no 3' polyadenosine overhangs (cf. Amplitaq®). The vector included in the kit is based on the pBluescript

(SK+) plasmid and contains the ampicillin resistance gene for antibiotic selection. The β-galactosidase gene is also present in the plasmid, and insertion of sequences into the vector at the *Srf*I restriction site causes disruption of this gene. When expressed, the β-gal gene encodes a protein which can metabolise the chromogenic substance X-gal, in the presence of IPTG, to give a blue pigmentation to the colony. Disruption of the β-gal gene abolishes expression of the protein and colonies remain white. The use of the β-gal gene in a plasmid therefore facilitates blue/white colony selection for presence of the inserted fragment.

Table II.vi Bacterial media

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

Following the manufacturers specifications, ligation reactions were performed in a final volume of 10µl. Reactions contained 10ng of cloning vector, 0.5mM rATP, 1x ligation buffer, 5U *Srf*I restriction enzyme, 4U T4 DNA ligase and 4µl of a tenfold dilution of PCR product. The reaction was incubated at room temperature for one hour and then heat inactivation of the enzymes was carried out by heating to 65°C for ten minutes.

The ligation reaction was transformed into Epicurian Coli XL1-Blue MRF' supercompetent cells (Stratagene) according to the manufacturers protocol. Bacterial cells were thawed on ice, the ligation reaction added and then incubated for 30 minutes in the presence of 25mM β-mercaptoethanol. After incubation the cells were heat-shocked for 45 seconds at 42°C and placed on ice for two minutes. Following transformation, the bacteria were incubated in SOC medium for one hour at 37°C in an orbital shaker to allow the cells to recover.

LB agar plates containing ampicillin at 100µg/ml were poured, allowed to solidify, and then dried at 37°C for 15-20 minutes. Plates were spread with 100µl X-gal (20mg/ml stock solution in dimethyl formamide) and 8µl IPTG (0.5M stock solution) to facilitate blue/white colony selection. As X-gal is dissolved in DMF, which is toxic, plates were treated with X-gal and IPTG at least 30 minutes prior to use to allow the DMF to absorb into the agar. The transformed bacteria were spread onto the prepared plates in 100µl, 150µl and 200µl volumes and the plates incubated at 37°C overnight. White and pale blue single colonies were chosen for further analysis, which is described in sections 2.2.5.2 - 2.2.5.5.

2.2.5.2 Small scale preparation of plasmid DNA

The chosen colonies were inoculated into 3ml cultures of LB-broth containing 100µg/ml ampicillin and grown overnight in an orbital shaker at 37°C. A volume of 1.5-2ml of each bacterial suspension was aspirated and the cells pelleted in a MSE microfuge at 13,000rpm for 30 seconds. Plasmid preparations were then performed using commercially available purification columns (Wizard Minipreps, Promega UK Ltd.), as follows.

The bacterial pellets were resuspended in cell resuspension buffer and subjected to sequential alkaline lysis and neutralisation using solutions provided within the kit. Bulk protein contaminants were removed from the solution by centrifugation in a microfuge at 13,000rpm for ten minutes. The clarified DNA suspensions were mixed with purification resin and passed through the Wizard purification columns under vacuum. The columns were washed twice and centrifuged briefly to dry the resin. Fifty microlitres of sterile nuclease-free water were added to each column and these were then centrifuged to elute the DNA.

2.2.5.3 Restriction endonuclease digestion of plasmids

Purified plasmid DNA was quantitated using a spectrophotometer (section 2.2.1.2) and 200ng of DNA were digested using *Pst*I and *Sst*I restriction enzymes. Five units of each enzyme were added to a 20µl reaction volume with 1x reaction buffer containing 10mM magnesium chloride and 50mM Tris pH 8.0 (REact® 1 buffer, Life Technologies). Incubation at 37°C for two hours allowed sufficient digestion of the plasmid to take place. Ten microlitres of the digest were electrophoresed on an 8% polyacrylamide gel alongside 0.4µg of ΦX 174 RF DNA digested with *Hae*III (Life Technologies), which was used as a size standard to facilitate estimation of DNA fragment size. The electrophoresis results confirmed the presence or absence of the inserted sequence.

2.2.5.4 Frozen bacterial stocks

Frozen bacterial stocks were prepared from cultures containing the desired plasmid construct.

Bacterial cultures were streaked onto L-agar plates containing ampicillin at a concentration of 100µg/ml and incubated at 37°C overnight. The following day, single colonies were picked and grown overnight in 2ml cultures of LB medium. Glycerol was then added to the cultures to 20% final concentration and the stocks stored as 1ml aliquots at -80°C. In this form the bacteria containing the cloned sequence can be stored indefinitely.

2.2.5.5 Nucleotide sequencing

Solutions used in the procedures described below are detailed in Table II.vii.

Preparation of plasmid DNA on a small scale gave sufficient material to perform nucleotide sequencing. DNA was prepared for sequencing utilising reagents from the Sequitherm™ Long-Read™ cycle sequencing kit for Li-Cor sequencers (Epicentre Technologies) (Wilson *et al.*, 1994).

Five hundred nanograms of each plasmid were used in a cycle sequencing reaction using universal primers M13Rev/IRD41 and M13Fwd(-29)/IRD41 (Li-Cor) which were complementary to vector sequences. The clones were sequenced using a Li-Cor DNA 4000 automatic sequencer which detects infra-red emissions; primers were labelled with infra-red dye which is extremely sensitive to light, therefore primers were handled in the dark. The sequencing reactions utilised a chain termination method, therefore four reactions were carried out per sample, one containing each dideoxynucleotide mix.

Five units of Sequitherm™ DNA polymerase were used in a reaction containing 1x reaction buffer, nucleotides, primer at 0.1-0.2pmol/µl and DNA template. Following an initial denaturation at 95°C for five minutes, thirty amplification cycles of 95°C for 30 seconds, 60°C for 30 seconds and 70°C for 60 seconds were performed before addition of termination mixes (Epicentre Technologies) for each nucleotide. Immediately prior to running the samples on the sequencing gel, stop solution/loading buffer (Epicentre Technologies) was added to each reaction. Samples were denatured by boiling at 95°C for five minutes to separate the synthesised fragments from the template DNA before being loaded onto the gel.

A 6% polyacrylamide sequencing gel was poured between glass plates designed to give a 20cm x 36cm gel, and allowed to polymerise for two hours. The gel was pre-electrophoresed at 1000V for one hour prior to loading the samples. Gel electrophoresis was carried out at 1000V for 6-8 hours during which time the sequence data were collected automatically onto an optical disk. The image was analysed at a later date.

Table II.vii Solutions for nucleotide sequencing

solution	*constituents*
10x reaction buffer	500mM Tris pH 9.3 25mM magnesium chloride
Stop solution/loading buffer	95% formamide 20mM EDTA 0.05% bromophenol blue 0.05% xylene cyanol
Li-Cor Long-Read™ sequencing gel	5% bis-acrylamide 1% acrylamide 8M urea 0.05% ammonium persulphate 0.05% TEMED
Running buffer	130mM Tris pH 8.5 @ 50°C 40mM boric acid 2.5mM EDTA

Table II.viii Solutions used in Western blot analysis

solution	*constituents*
Sample lysis buffer	50mM Tris pH 8.0 0.15M sodium chloride 0.1% SDS 0.5% Sodium deoxycholate 1% NP40
2x sample loading buffer (reducing)	12.5mM Tris pH 8.8 40% glycerol 4% SDS 10% β-mercaptoethanol 0.04% bromophenol blue
Protein resolving gel	15% Easigel 37.5:1 acrylamide mix 0.375mM Tris pH 8.8 0.1% SDS 0.1% ammonium persulphate 0.04% TEMED
Protein stacking gel	5% Easigel 37.5:1 acrylamide mix 125mM Tris pH 6.8 0.1% SDS 0.1% ammonium persulphate 0.1% TEMED
SDS-PAGE running buffer	25mM Tris pH 8.3 250mM glycine 0.1% SDS
Semi-dry transfer buffer	48mM Tris 39mM glycine 0.01% SDS 20% methanol
Marvel blotting buffer	3-5% Marvel 0.5% Tween 20 TBS pH 7.6

2.2.6 Western blotting

Details of stock solutions, buffers and gel compositions are presented in Table II.viii.

2.2.6.1 Preparation of cell lysates for Western blotting

Cells were washed twice in 1x PBS and pelleted prior to lysis. The pellets were resuspended in an appropriate lysis buffer at a concentration of $1.3x10^8$ cells/ml. Protease inhibitors were included in this buffer, for example 100μg/ml PMSF, 10μg/ml aprotonin and 10μg/ml leupeptin. The cell suspensions were vortexed and incubated on ice for 15 minutes. The lysates were then centrifuged to remove debris and the supernatants harvested. Protein suspensions obtained in this way were mixed with an equal volume of sample loading buffer and snap frozen in liquid nitrogen before being stored at -80°C until use.

2.2.6.2 SDS-PAGE for protein separation

Proteins were separated using 15% denaturing polyacrylamide gels with a 5% polyacrylamide stacking gel. The resolving gels were poured between glass plates designed for the Bio-Rad Mini-Protean II system which resulted in gels sized 10cm x 6cm x 0.1cm with a 10cm x 1cm x 0.1cm stacking gel. Sample lysates (section 2.2.6.1) were thawed and then boiled for five minutes to ensure the proteins were denatured. Samples were then loaded onto the stacking gel at $1x10^6$ cell equivalents per lane. Electrophoresis was carried out by initially applying 150V for 15 minutes to allow the proteins to stack; after this step the voltage was increased to 200V for 45 minutes.

2.2.6.3 Western blotting

The SDS-PAGE resolving gels were soaked in transfer buffer for ten minutes prior to blotting. The membranes (Immobilon P; Millipore UK Ltd.) were placed in 100% methanol before being soaked in transfer buffer alongside the gels. The membranes and gels were aligned and loaded into a Semidry Transblotter (Bio-Rad Laboratories) between two sheets of extra thick filter paper (Bio-Rad Laboratories) soaked in transfer buffer. Blotting was achieved by applying 15V across the apparatus for 40 minutes.

After blotting, the membranes were equilibrated briefly in TBS pH 7.6 before being placed in a solution of 5% Marvel buffer (Table II.viii) for 30 minutes. This ensured blocking of non-specific binding sites. All incubations were carried out at room temperature on an orbital shaker. Following blocking, the buffer was removed from the blots and primary monoclonal antibody added at a concentration of 1μg/ml in 3% Marvel buffer. All subsequent steps were performed in this buffer. The blots were incubated with the antibody for two hours, after which time the solution was removed and the membranes washed three times in excess volumes of Marvel buffer for ten minutes each wash. The blots were then incubated for 90 minutes with secondary antibody, conjugated to horseradish peroxidase (Amersham International PLC), diluted 1:3000 in Marvel buffer. The membranes were subjected to three washes of ten minutes in TBS pH 7.6, 0.5% Tween20. Omission of Marvel was necessary in the final washes as the milk proteins contain biotin which interferes with the detection process.

The blots were developed using the Amersham ECL Western blotting protein detection reagents. The ECL system is based on the emission of chemiluminescence from the combined detection reagents. The horseradish peroxidase, conjugated to the secondary antibody, oxidises an enzyme present within the ECL detection kit reagents. This then catalyses a reaction involving luminol and a phenolic enhancer compound under alkaline conditions. The result is luminol in the excited state; the reaction peaks after 5-20 minutes and the 428nm wavelength emissions generated can be detected by short exposure to blue-light-sensitive autoradiographic film. Hyperfilm-ECL (Amersham International PLC) was used for this procedure and exposure times of 30 seconds, two minutes and five minutes were used.

2.2.7 Immunohistochemical assays

2.2.7.1 Coating slides with APES

Microscope slides were coated with APES to enable greater adherence of cell preparations and tissue sections. This was important for use in the immunohistochemical procedures detailed below, as the slides were subjected to lengthy incubations and numerous washes.

Glass microscope slides were soaked overnight in 10% Decon (Fisons Ltd.) at room temperature. They were then washed in running tap water and rinsed in distilled water to remove any traces of Decon. Slides were dried overnight at 60°C. Once cool the slides were immersed in a 2% v/v solution of APES (Sigma Chemical Company) in acetone for five seconds, rinsed briefly in acetone alone and then washed with distilled water (Maddox & Jenkins, 1987). The slides were dried overnight at 37°C and stored at room temperature until use.

2.2.7.2 Dewaxing paraffin-embedded tissue sections

Paraffin-embedded sections of biopsy tissue 3-5μm thick were mounted on APES-coated microscope slides (section 2.2.7.1). The sections were dewaxed for a minimum of ten minutes in two changes of a xylene substitute e.g. Citroclear (National Diagnostics), and then rehydrated in an ethanol gradient as follows. The slides were placed in 100% ethanol for two minutes, 70% ethanol for two minutes and methylated spirit for a further two minutes before being rinsed in clean water. They were then immersed in Lugel's iodine for two minutes to remove any traces of mercuric fixatives, placed in clearing solution (5% w/v sodium thiosulphate) to remove iodine/mercury complexes, and then rinsed in distilled water. The sections were equilibrated in TBS pH 7.6 for five minutes prior to immunohistochemical analysis.

2.2.7.3 Preparation of samples

Paraffin-embedded tissue sections were dewaxed as described in section 2.2.7.2 and equilibrated in TBS pH 7.6. Cell cytospins which had been stored at -20°C were allowed to reach room temperature before being equilibrated in TBS pH 7.6 for a minimum of ten minutes.

2.2.7.4 Avidin-biotin complex (ABC) immunohistochemistry

Cytospin preparations used in this procedure were fixed in ice-cold acetone for at least 15 minutes and air dried. Paraffin sections were pretreated as described in section 2.2.7.2. At no time during the described procedure were the sections allowed to dry out as this produced staining artefacts. All steps were performed at room temperature unless otherwise stated.

The slides were immersed in TBS pH 7.6 containing 0.1% BSA (TBS buffer) for ten minutes and then microwaved for ten minutes in 0.01M tri-sodium citrate buffer at pH 6.0 to unmask antigens. The slides were rinsed briefly in distilled water and returned to TBS buffer for five minutes. The sections were incubated with a solution of heat-inactivated normal rabbit serum (SAPU), diluted to 20% in TBS buffer, for 30 minutes, which served to block any non-specific binding of antibody.

The blocking buffer was removed and primary antibody was added to the sections at an appropriate dilution in TBS buffer and incubated overnight at 4°C. After incubation, sections were washed for 2 x 5 minutes in TBS buffer and the secondary antibody added to the slides at a dilution of 1:200 in TBS buffer. The secondary antibody used was biotinylated rabbit anti-mouse immunoglobulins (Dako Ltd.). The sections were incubated with the antibody for 30 minutes and then washed for 2 x 5 minutes in TBS pH 7.6. ABC-alkaline phosphatase (AP) complex (Dako Ltd.) was added to the sections at a dilution of 1:50 in TBS pH 7.6 and incubated for 30 minutes. The ABC-AP suspension was prepared 30 minutes prior to use to enable complex formation to take place. Following incubation the sections were washed in TBS pH 7.6 for 2 x 5 minutes. Fast red TR salt (Sigma Chemical Company), the substrate for the reaction, was diluted in AP substrate buffer (Table II.v) to 1mg/ml and this was filtered through Whatman No.1 filter paper (Fisons Ltd.) and incubated with the slides for 20 minutes.

The sections were rinsed briefly in water and counterstained in Mayer's haematoxylin before being mounted using an aqueous mountant (Aquamount, BDH (Merck Ltd.)).

2.2.7.5 Alkaline phosphatase anti-alkaline phosphatase (APAAP) immunohistochemistry

The procedure used was essentially the same as that described in section 2.2.7.4 until the addition of the secondary antibody. For the APAAP method the secondary antibody is a bridging antibody and the antibody used was biotinylated rabbit anti-mouse immunoglobulins (Dako Ltd.). This was added to the slides at a dilution of 1:25 in TBS buffer and incubated for 30 minutes. The sections were then washed in two changes of TBS pH 7.6 for ten minutes. APAAP complex at a 1:50 dilution in TBS pH 7.6 was incubated with the sections for a further 30 minutes, and the slides washed in TBS for ten minutes. The secondary antibody and APAAP incubations were repeated, with washes, for 15 minutes each before staining with Fast red TR salt and mounting as described in section 2.2.7.4.

Chapter 3

Use of the *in vitro* regression assay to investigate EBV-specific cell-mediated immune responses in Hodgkin's disease

3.1 Introduction

EBV is a common pathogen with over 90% of the population seroconverting in the first decade of life. The immune response to EBV has two main components. The first, or primary, response is mounted at the time of initial infection with the virus, and is important in containing the virus and limiting its spread. This response plays a part in determining the eventual viral load of EBV that is carried throughout life. The second component is the memory immune response. This is important in maintaining the host-virus equilibrium and it acts to control the extent of virus reactivation events that result in the production of extracellular infectious virus. CTLs function in both the primary and memory immune responses.

Neutralising antibodies are generated against viral antigens, including EA and VCA, as part of the primary immune response to EBV (De The, 1982). This antibody response plays a major role in the control of initial virus spread. Presence of neutralising antibodies is therefore a factor in the determination of the eventual persistent viral load in peripheral blood and lymphoid tissue. Longterm antibody titres to EBV VCA can be used as an indirect indicator of viral load (Henle & Henle, 1979).

Initial virus load can be an important determinant of the outcome of a persistent viral infection. For example with HTLV-1 infection high antibody titres to the virus correlate with a high viral load, and are associated with conditions resulting from immune mediation of infection e.g. tropical spastic paraperesis (Bangham, 1993; Bangham *et al.*, 1996).

CTLs, particularly of the memory immune response, are known to be a major influence in the control of viral infections (Nowak & Bangham, 1996). In the case of HTLV-1 there is usually a vigorous CTL response to the virus in seropositive people; however, patients with a low CTL response develop the highest viral load (Bangham *et al.*, 1996). For EBV, it has been shown that there are virus-specific CTLs which respond to peptide epitopes of viral latent and lytic proteins presented by MHC class I molecules (reviewed by Kurilla, 1993). Removal of CTL immune surveillance can result in reactivation events occurring unchecked, and to the frequent development of EBV-driven lymphoproliferative disease. When immunosuppression is removed, regression of such disease states usually occurs (Starzl *et al.*, 1984). It can thus be concluded that CTL control of EBV infection is vital to the establishment of a lifelong virus-host relationship.

Raised antibody titres to EBV in HD patients were reported in several studies in the 1970s, initiating the theory that there was an association between the virus and the disease (Levine *et al.*, 1971; Henle & Henle, 1973; Evans *et al.*, 1978). Since then a subgroup of HD cases which are associated with EBV has been identified using molecular biology techniques (section 1.5.1). It is now clear, however, that raised antibody titres to EBV antigens occur in both EBV-associated and non-EBV-associated cases of HD. Serology does not provide an indication of EBV status of RS cells, but as a group EBV-associated HD cases display higher titres to VCA than non-associated cases (D.Clark, unpublished results). These findings provide indirect evidence that HD patients, particularly those with EBV-associated disease, have a high EBV viral load prior to disease onset. It can therefore be postulated that high EBV viral load may be a risk factor for the development of HD.

Increased viral load is likely to be accompanied by an increased number of EBV-infected cells. This would increase the chance of an RS cell precursor becoming infected with EBV, both directly and by increasing the numbers of reactivation events occurring. The existence of larger numbers of infected RS cell precursors would increase the chances of one of these acquiring other mutations necessary for tumour development. As little is known about the exact role played by the virus in EBV-associated HD, either of these possible consequences of high viral load may contribute to the pathology of these cases.

RS cells in approximately 40% of HD cases carry EBV genomes and express a restricted subset of viral gene products, namely EBNA-1 and the LMPs (Pallesen *et al.*, 1991; Deacon *et al.*, 1993; Grasser *et al.*, 1994), which is referred to as a type II pattern of latency or Lat II (section 1.4.6). The LMPs are immunogenic proteins and several peptide epitopes of these proteins have been shown to elicit an HLA class I-restricted response (Khanna *et al.*, 1992; R.J.Murray *et al.*, 1992). This raises important questions with regard to EBV-associated HD, where the RS cells have been shown to express LMP-1 on their surface. LMP-2 expression has been demonstrated at the mRNA level, and is presumed to be expressed as protein; this has not been confirmed as there are no good monoclonal antibodies to LMP-2 available at the present time. The expression of these viral proteins suggests that RS cells should be a target for lysis by class I-restricted CTLs. However the RS cells persist, suggesting that an underlying impairment of cell-mediated immunity to EBV might be present in people who develop EBV-associated HD.

Abnormalities of T-cell mediated immune function have been reported in HD patients (reviewed by Slivnick *et al.*, 1990). These abnormalities include reduced CTL proliferation in response to soluble antigen (Levy *et al.*, 1974; Holm *et al.*, 1976), lymphocytopoenia (Matchett *et al.*, 1973), distorted ratios of T-cell subpopulations (Gupta, 1980) and increased susceptibility to certain types of infection (Casazza *et al.*, 1966). These defects can persist in HD patients who have entered remission (Fisher, 1982). In non-Hodgkin's lymphomas (NHL), secondary immuno-suppression is a common feature. However in NHL, abnormalities of cell-mediated immunity occur mostly in advanced disease states, whereas in HD these abnormalities occur early in disease development (Kumar & Penny, 1982). There are also documented cases of defective CTL function occurring in first order relatives of HD sufferers (Del Giacco *et al.*, 1985; Merk *et al.*, 1990). These separate lines of evidence support the suggestion that immune dysfunction precedes onset of disease rather than being secondary to tumour development.

In summary, the CTL immune response to EBV is a major component of both the primary and memory response. Decreased efficiency of the "primary" CTLs may result in high viral load, which in turn may predispose to the onset of EBV-associated HD. The memory immune response, namely CTLs, should provide immunity to EBV-related tumour development throughout life regardless of viral load. Reactivation events are normally recognised and contained. Latently infected B-cells expressing immunogenic viral antigens should also be recognised and eliminated. Defects in the CTL arm of the memory immune response may therefore also be a critical determining factor in the development of EBV-associated malignancies including EBV-associated HD.

The experiments described in this chapter investigated whether a general defect in EBV-specific cell-mediated immunity is a feature of HD. The method used to investigate this was an *in vitro* technique which measures the ability of memory CTLs in the peripheral blood to regress the outgrowth of EBV-transformed B-cells (Moss *et al.*, 1978). The original study describing the assay used it to investigate long-term T-cell-mediated immunity to EBV in healthy individuals. Since then it has been implemented in a number of studies, including investigations into alterations of the virus-host balance in post-transplant patients and in IM patients receiving antiviral therapy (Yao *et al.*, 1985; 1989a).

The assay, termed *in vitro* regression (IVR) assay, was developed to allow quantitative comparisons of cell-mediated immunity to EBV between individuals. PBMCs from test donors are obtained and, in order to stimulate EBV-specific T-cell responses, PBMCs are infected with EBV using high concentrations of B95-8 viral stock *in vitro*. The strength of any EBV-specific CTL response can then be measured by recording the ability of the test donor's T-cells to recognise and lyse EBV-transformed B-cell outgrowths within the cultures. This results in "regression" of the transformed outgrowths. Following infection with EBV, PBMCs are cultured at a series of twofold dilutions of cell number. Regression is strictly cell concentration-dependent, and is most readily observed at high cell concentrations. Thus the "strength" of regression of an individual, representing the effectiveness of EBV-specific CTLs, can be assayed and expressed as the minimum initial cell concentration required for 50% of replicate cultures to show regression (Moss *et al.*, 1983). This is an endpoint value which can be used for comparisons between individuals (section 3.3.2).

In 1981, IVR assays were performed on the peripheral blood of 54 patients with NPC (Chan & Chew, 1981). All patients and controls (n=38) included in the study were EBV seropositive. It was found that there was no, or only partial, regression of EBV-transformed B-cells in NPC patients, whereas in controls there was complete regression in most cases. It was concluded from this study that there was a marked deficiency in the generation of EBV-specific CTLs *in vitro*, and it was postulated that this could be a factor in the persistence of tumour cells in NPC patients.

This study is of particular relevance to the work of this chapter, as there are several similarities between NPC and HD. The pattern of viral latent gene expression is the same for NPC tumour cells and RS cells of HD; both express a Lat II phenotype. There is a large cellular infiltrate present in NPC tumours as with HD lesions. As described above, impairment of cell-mediated immunity to EBV is implicated in the pathogenesis of NPC and is suspected in HD. Both diseases display varying degrees of familial clustering, and a proportion of cases are HLA-associated (Chan *et al.*, 1983; Lu *et al.*, 1990; Burt *et al.*, 1994); some epidemiological studies suggest a similar association for this disease (Amiel, 1967; Bodmer *et al.*, 1989).

In these experiments we investigated the possibility of decreased strength of regression occurring in HD patients relative to healthy control individuals.

3.2 Materials & methods

3.2.1 Case selection

Peripheral blood samples were collected from HD patients in the West of Scotland and Northern Region of England. At the time of setting up the assays, EBV status of HD cases was not known. Pretreatment cases were chosen exclusively for this study to nullify any effects of treatment on the possible outcome of the investigation. Age and sex matched healthy controls were collected, where possible, and assayed alongside cases. Seropositive and seronegative control samples were also included in experiments. In total, results were obtained from 13 HD cases and nine controls.

3.2.2 Sample preparation

Samples of peripheral blood were received as described in section 2.1.1.1. PBMCs were isolated from these samples using a Ficoll-paque density gradient as outlined in section 2.1.1.2. In some cases PBMCs were stored frozen in liquid nitrogen (section 2.1.1.5) prior to the IVR assay.

3.2.3 Determination of EBV status

Sections of paraffin-embedded lymph node biopsies, mounted on APES-coated slides (section 2.2.7.1), were used for EBER *in situ* hybridisation analysis. Sections were hybridised with a biotinylated oligonucleotide probe specific for the EBV EBER-1 RNA, which is abundantly transcribed in cells latently infected with EBV (Weiss *et al.*, 1991; Armstrong *et al.*, 1992). In later experiments EBER RNA expression was examined using commercially prepared FITC-conjugated EBER oligonucleotide probes (Dako Ltd.). The hybridisation signal was detected using an alkaline phosphatase conjugated antibody system utilising nitroblue tetrazolium as chromogenic substrate. A known positive sample was included in each assay and a biotinylated nonsense oligonucleotide probe was used as a negative control. EBER *in situ* hybridisation was carried out by Alison Armstrong and Diane Gray.

3.2.4 EBV serology

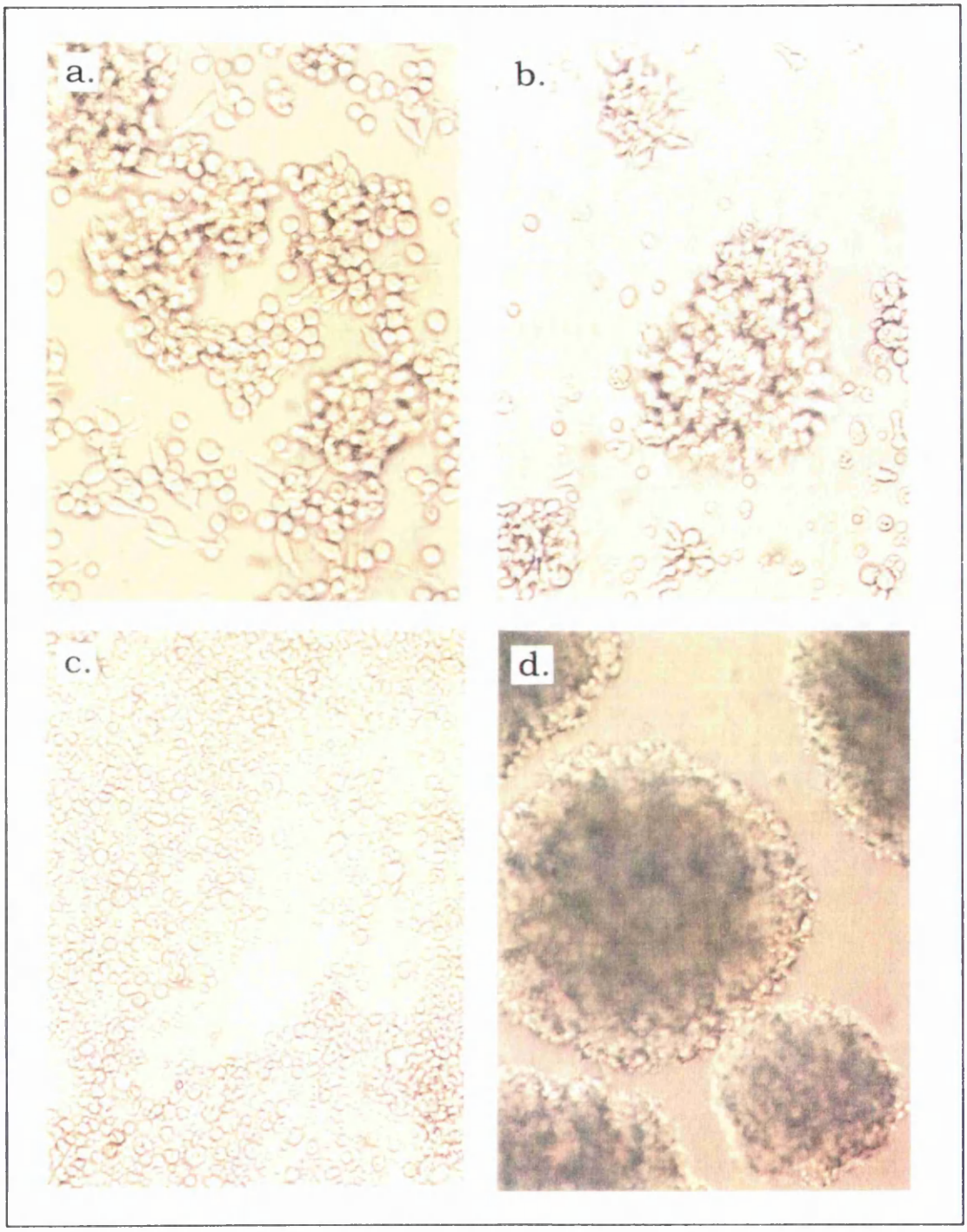
Antibody titres to EBV VCA and EA were determined using indirect immunofluorescence assays (IFA). The primary aim of this aspect of the study was to determine whether individuals were EBV seropositive. All sera were initially screened at a dilution of 1:10. Positive sera were diluted two-fold further until an end titre was reached, defined as the reciprocal of the serum dilution at which specific fluorescence was last observed. VCA IFA utilised B95-8 cells as a source of antigen, according to the method first described by Henle & Henle (1966). Diffuse and restricted components of EBV EA were assayed in an IFA based on that of Long *et al.* (1974), either utilising Raji cells, treated with 5-iododeoxyuridine, as antigen, or using commercially available slides (Gull Laboratories Inc.). EBV serology was performed by Diane Gray.

3.3 *In vitro* regression assay

The principle of this assay is based on the ability of EBV-specific CTLs to regress the outgrowth of EBV-transformed autologous B-cells in primary lymphocyte cultures. Mononuclear cells from peripheral blood are infected with EBV to facilitate outgrowth of virus-transformed B-cell foci. Cells at six hours post infection are illustrated in Fig.3.1a. The appearance of transformed B-cell clumps, seen 7-10 days post infection, is shown in Fig.3.1b. The transformed B-cells express a Lat III phenotype and are recognised by CTLs in the culture which are specific for EBV latent antigens. CTL lysis of EBV-infected B-cells occurs, and is seen in the cultures as regression of the clumps of transformed cells; the clumps appear to disintegrate. Eventually complete regression of B-cell outgrowths can be seen (Fig.3.1c). In the absence of an EBV-specific CTL response, or in the presence of cyclosporin A (CsA) which inhibits T-cell function, regression is not seen and large LCL-like clumps of latently-infected, transformed cells can be seen (Fig.3.1d). The end result of the assay is scored at four weeks post infection.

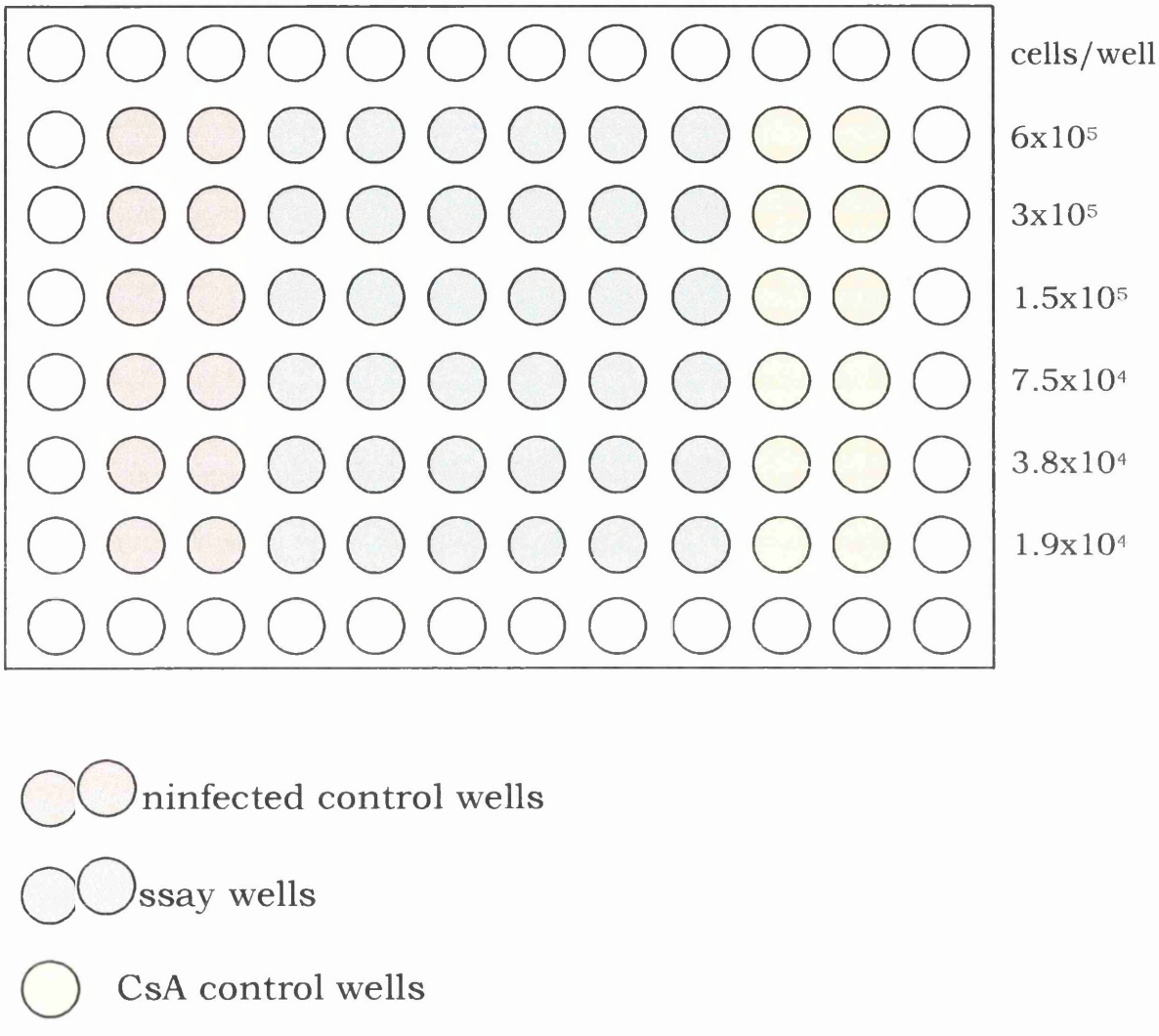
The IVR assay is carried out in a 96-well microtitre plate (Fig 3.2). The assay lanes consist of 6 replicate wells of $6x10^5$, $3x10^5$, $1.5x10^5$, $7.5x10^4$, $3.8x10^4$, and $1.9x10^4$ cells per well. The controls for the assay include two replicate wells of uninfected cells at each cell concentration and two replicate wells of infected cells in the presence of CsA at a concentration of 0.1μg/ml. The uninfected control lanes are necessary to exclude the possibility that spontaneous transformation has occurred. The CsA control is included as an indicator of the transformation efficiency of the viral stock. Replicates of any cell concentration are scored only if the cells in the CsA control wells are transformed and those in the uninfected control wells are dead.

Fig.3.1 Appearance of cells during the IVR assay



a. cells at six hours post infection
b. transformed outgrowths of cells after 7-10 days
c. regression of EBV-transformed B-cell clumps seen after 2-3 weeks
d. LCL-like growth of non-regressed B-cells seen after 3-4 weeks

Fig.3.2 Layout of the IVR assay



3.3.1 Protocol for setting up the IVR assay

PBMCs used in this assay were either freshly isolated or thawed from storage in liquid nitrogen (section 2.1.1.5). The cells to be infected with EBV were resuspended in 300µl of concentrated viral stock (thawed at 37°C) and the volume made up to 600µl with complete medium. The cells for the uninfected control were resuspended in 600µl of complete medium alone. Cell suspensions were incubated at 37°C with 5% CO_2 for one hour with occasional agitation to ensure that the cells remained in suspension.

Following incubation the cells were washed, resuspended at $6x10^6$ cells/ml and serial doubling dilutions performed. At this point the cells for the CsA control lanes were aliquotted into wells of the microtitre plate and CsA was added. Cells were plated out in 200µl of medium, which was made up to a total of 300µl per well with complete medium.

In cases where there were too few cells to set up a complete assay, the assay was scaled down within certain limits. The initial step was usually to use $3x10^5$ cells per well (cf. $6x10^5$) as the highest cell concentration. Where necessary the assay was further limited by including only one lane of each control and reducing the number of replicate assay wells at each dilution from six down to four or two. The latter reduction was necessary in only one case, the seronegative control.

3.3.2 Scoring the IVR assay

Scoring the assay lanes involves recording the occurrence of transformation or regression for each of the wells. A formula (Appendix C) is used to give a value for the minimum initial cell number required to give a 50% incidence of regression (Reed & Meunch, 1938). This value can then be compared among individuals assayed.

The normal range of strengths of regression for healthy carriers is defined as minimum initial cell concentrations of $0.4x10^5$ - $6x10^5$ cells/well (Rickinson *et al.*, 1981; Yao *et al.*, 1985). Within this range, regression at the higher cell concentrations, $3x10^5$ - $6x10^5$ is accepted as being at the weaker end of normal (Yao *et al.*, 1985).

3.4 Development of the assay

It is essential that a very high transformation efficiency is attained in order to be able to score the result of this assay. Difficulties were encountered due to sub-optimal transformation and to cell death occurring in the second week of the assay. Extensive optimisation experiments were therefore performed, using control PBMCs from healthy donors, prior to running the assay on HD samples. The optimisation experiments described were carried out contemporaneously.

3.4.1 Preparation of concentrated B95-8 EBV stocks

Originally the virus used was prepared as described in "Lymphocytes - A Practical Approach", Chapter Seven (Walls & Crawford, 1987). The EBV stocks consisted of neat tissue culture supernatant harvested from TPA-stimulated B95-8 cells five days post-stimulation. TPA stimulation was performed as it increases the quantity of infectious virus available in the supernatant as a consequence of lytic replication. Use of this viral stock resulted in inefficient transformation and the assay could not be scored.

Initially virus was prepared from B95-8 stock cell lines stored within the department. Due to the inefficiency of transformation described above, a new stock of B95-8 cells was obtained from Dr. Q-Y. Yao at the CRC Laboratories, Birmingham University Medical School. These cells had different growth characteristics from our original stock, growing in larger clumps and to greater density. The new batch of cells was grown as described above and the supernatant harvested and titrated on CBMCs (section 2.1.2.2). The transformation efficiency of this virus was too low to give an accurate titre. However, in all subsequent optimisations these B95-8 cells were used in place of the original batch of cells.

Following these observations, the TPA-stimulated B95-8 supernatant containing the viral particles was harvested, as described above, and concentrated 100-fold by ultracentrifugation (section 2.1.2.1). The viral stocks were titrated using CBMCs as each new batch was prepared (section 2.1.2.2).

The efficiency of transformation improved greatly following the use of concentrated virus and at one week post-infection all the infected assay wells showed evidence of outgrowth of transformed B-cells, i.e. clumps of cells were present in all the infected wells and absent from the uninfected controls. However, these clumps did not increase in size or number and, after 2-3 weeks, cells within the clumps became dark and granular in appearance, and eventually no viable clumps remained.

Cells in the uninfected wells also died during the course of the assay, but the appearance of these cells was different from that of the infected cells. Some cells clumped after initial plating out (depending on the donor) and some remained as single cells. Cell death occurred at around 3-4 weeks with disintegration of cells and appearance of transparent debris and cell ghosts.

Due to the above observations, it was hypothesised that the viral preparation must in some way be causing the cell death seen in the infected wells. Consequently the viral harvest method was again scrutinised. It is known that TPA, which was present in the B95-8 tissue culture supernatant at 20ng/ml, is toxic to cells at high concentration. It was originally thought that as TPA is a small molecule it would not be concentrated on centrifugation along with the virus. It is also known that TPA associates with cell membranes, and as an extrapolation of this it was suggested that the TPA was associating with the envelope of the virus particles and being concentrated along with the virus during ultracentrifugation. Thus it was proposed that a toxic concentration of TPA was present in the viral preparations.

To test this possibility, viral suspensions were harvested from unstimulated B95-8 culture supernatant and concentrated as described in section 2.1.2.1. Cultures were grown at 35°C as this is known to increase virus production from the cells.

Assays were set up using all the optimisations described above and using the viral stock prepared without the use of TPA. The results were noticeably different from the previous assays. One week post-infection the infected wells contained numerous small clumps of transformed B-cells. The cells within the clumps were bright and refractile and cytoplasmic protrusions could be seen at the surface of the clumps. The clumps increased in size and number up to the two week point and still appeared healthy in the assay wells.

3.4.2 Presence of contaminating organisms

Experiments were carried out to determine whether bacteria or mycoplasma were present, as these organisms could have an effect on the survival of long-term cultures.

MRC-5 cells were seeded into slide flasks and allowed to reach confluence. At this stage 1ml of medium taken from the assay wells and CsA control wells at one, two and four week time-points was inoculated onto the monolayers. Bacterial contamination can cause disruption of the monolayer and peeling off of the cells; mycoplasma in the fibroblasts can be visualised using Hoescht staining (section 2.1.3.3).

The cultures were negative for mycoplasma at all times and were clear of bacteria at all except the four week time point. It was concluded that the death of the cultures could not be attributed to the presence of either organism.

3.4.3 General culture conditions

The culture conditions used in the assay were scrutinised for ways to prolong cell viability and promote transformation. Several parameters were investigated during the course of these optimisations.

3.4.3.1 Effect of humidity

It was noted that after two weeks in culture the volume of medium in the outermost wells of the microtitre plate had decreased. In order to minimise this the outside empty wells were filled with complete medium to maintain humidity across the plate. This was indeed found to decrease the loss of volume in the outside wells. In addition to this, cells were plated out in 300μl of medium per well in place of the initial 200μl. The individual and combined effects of these changes did maintain a more even humidity throughout the duration of the assay.

3.4.3.2 Effect of pH

The pH of the cultures, as indicated by phenol red dye present in the RPMI 1640 medium, was noted to be high from early in the assay through to the end point at four weeks. In order to investigate the effect of addition of HEPES buffer (Table II.i), an experiment was set up in duplicate using PBMCs from a single donor. Presence of HEPES maintained the culture at a lower pH, however, this modification did not alter the overall success of the assay and was subsequently omitted.

3.4.3.3 Effect of CO_2 concentration

Following improvements in the production of viral stocks, concern was again directed at the high pH of the culture medium. Concentration of CO_2 was maintained at 5% throughout the development of the assay using a Heraeus incubator which operated with high humidity. Scrutinisation of the incubator led to the discovery that the instrument had been wrongly calibrated. Although it was reading 5% CO_2 it was actually running at approximately 3.5% CO_2. Following rectification of the fault, assays were sustainable for the four week period required for scoring.

3.4.4 Conclusions

In retrospect the assay was initially failing for several reasons. The most critical factors overall being the presence of TPA from the virus preparation step and the high pH of the culture medium. When both these factors were identified and rectified the assay had a much greater frequency of success; however other factors also affected the performance of the assay although to a lesser degree.

3.5 Results

3.5.1 Serology and EBV status

Serology was performed for all 23 individuals in the study and the results are given in Table III.i. All individuals included in the study were EBV seropositive, with the exception of two seronegative individuals included as controls. These results are part of a larger HD data set which shows raised EBV titres in cases of EBV-associated HD as a group, as compared to non-associated cases.

EBV status of the 13 HD cases, as determined by EBV-EBER *in situ* hybridisation analysis, is also given in Table III.i. An example of a positive result, showing dark staining localised to the nuclei of RS cells, is shown in Fig.3.3.

3.5.2 Results of IVR assays

The end results of the IVR assays fall into three main categories as follows. First, there were those which did not show regression at all, with transformation persisting in all wells for the duration of the assay. An example of this group of cases is shown in Fig.3.4a. At the end of the four weeks, cells in the uninfected control wells were dead. Cells in the CsA control wells were transformed and LCL-like outgrowths could be clearly seen in all the wells. In the assay wells, transformation was also seen in all the wells. Eight cases, including two cases of EBV-associated HD and five controls, came into this category. As expected, neither of the seronegative controls showed regression.

Secondly, there were cases in which varying degrees of incomplete regression occurred. Assays for five HD cases, one of which was EBV-associated, and four controls behaved in this way. An example of partial regression is shown in Fig.3.4b.

Thirdly, the occurrence of complete regression was seen. The uninfected control wells and the CsA control wells were as described for "non-regressers". Complete regression was seen in the assay wells. One assay, which was subsequently used as a positive control, came into this category. The appearance of this assay at the four week time point is shown in Fig.3.4c.

Table III.i Serology results and EBV status for cases and controls

HD cases:

patient no.	*EBV status*	*VCA titre*	*EA titre*
3200	EBV+	2560	40
3248	-ve	1280	160
3249	-ve	5120	160
3250	-ve	1280	160
3251	-ve	1280	40
3277	EBV+	2560	320
3292	-ve	2560	80
3543	ND*	seropositive	
3619	-ve	seropositive	
3763	-ve	80	0
3790	-ve	5120	1280
3886	EBV+	5120	40
3888	-ve	20480	40

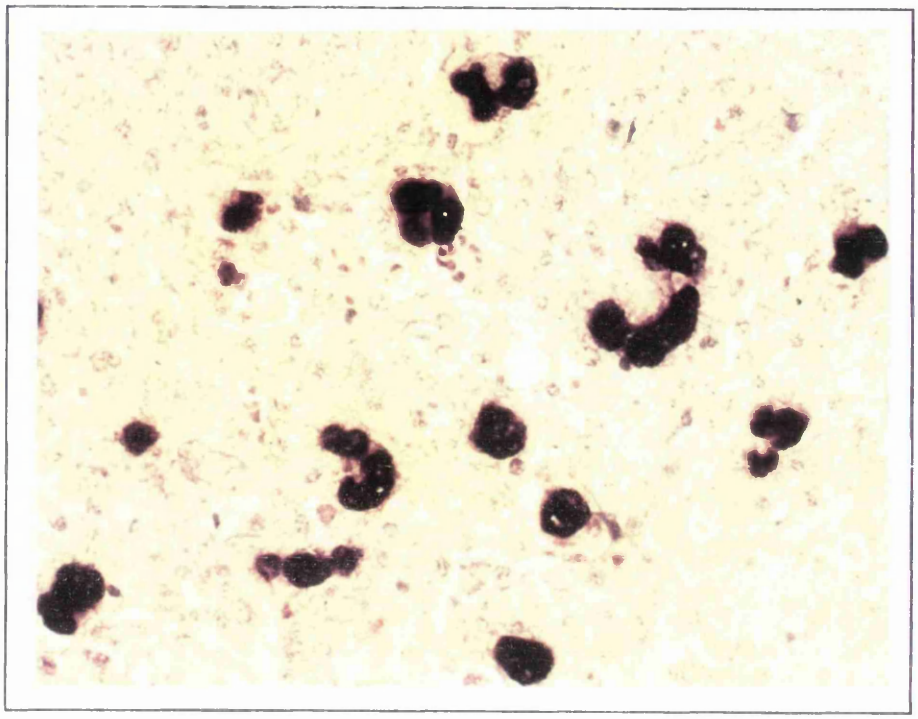
*ND = not done

seropositive = tested at 1:10 dilution, no further titration performed

controls:

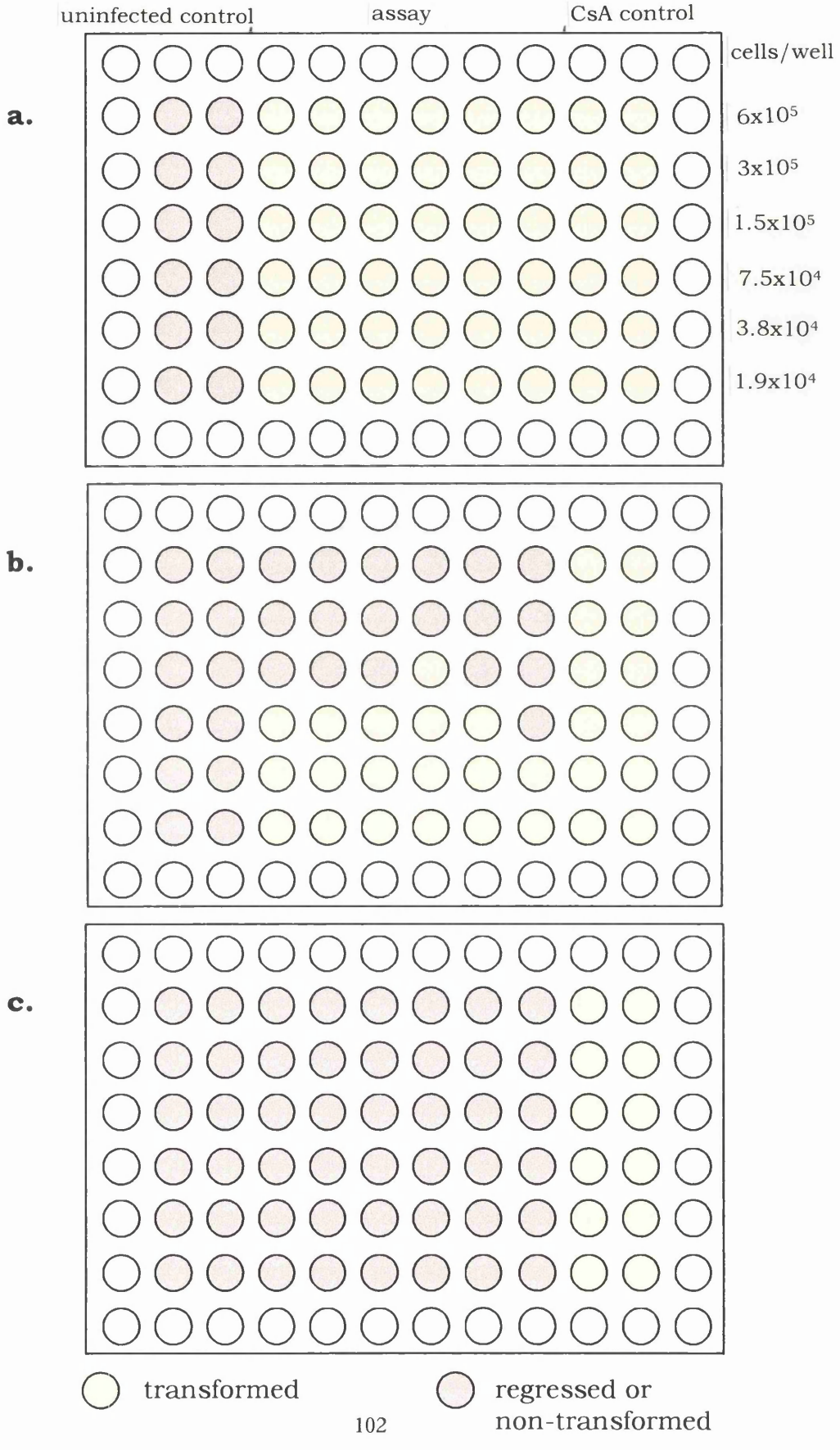
patient no.	*VCA titre*	*EA titre*
3213	2560	10
3258	2560	0
3260	40	0
3261	10240	80
3272	640	20
3788	2560	80
3893	seropositive	
3894	seropositive	
neg. 1	seronegative	
neg. 2	seronegative	

Fig.3.3 EBER *in situ* hybridisation of EBV+ case



Appearance of a positive result of EBER *in situ* hybridisation, showing dark staining localised to the nuclei of RS cells (x200)

Fig.3.4 Diagrammatic representations of IVR assays



A summary of the values for minimum cell concentration required for 50% incidence of regression, scored at four weeks post infection, is given in Table III.ii. The microtitre plate scores for regression and transformation of each well, plus calculations of the 50% endpoints for each assay, are given in Appendix C.

Five of thirteen HD cases showed regression within the normal range, defined as minimum initial concentrations of $0.4x10^5$ - $6x10^5$ cells/well (Rickinson *et al.*, 1981; Yao *et al.*, 1985b) (section 3.3.2), compared to five of eight controls. With such a small study it is not possible to assess the significance of the observed difference. One of three EBV-associated HD cases displayed regression, compared to four of ten non-EBV-associated cases. Minimum initial cell number for 50% regression for the EBV-associated case was $3.3x10^5$, which is at the weaker end of the normal range (Yao *et al.*, 1985b). The non-EBV-associated HD cases which showed regression were all at the stronger end of the normal range, with values of $1\text{-}1.9x10^5$.

Table III.ii Results of IVR assays

HD cases:

patient no.	*sex/age*	*EBV status*	*regression*	*50% endpoint*
3248	F38	-ve	part	$1.1x10^5$
3250	F65	-ve	part	$1.1x10^5$
3277	M59	EBV+	part	$3.3x10^5$
3292	M24	-ve	part	$1x10^5$
3888	F33	-ve	part	$1.9x10^5$
3200	F61	EBV+	none	
3249	F68	-ve	none	
3251	F36	-ve	none	
3543	M20		none	
3619	M35	-ve	none	
3763	M28	-ve	none	
3790	F65	-ve	none	
3886	F20	EBV+	none	

controls:

patient no.	*sex/age*	*regression*	*50% endpoint*
3213	M46	total	$<0.2x10^5$
3258	F26	part	$1.2x10^5$
3788	M30	part	$1.5x10^5$
3893	F33	part	$0.9x10^5$
3894	F26	part	$0.9x10^5$
3260	M21	none	
3261	M40	none	
3272	M37	none	

3.6 Discussion

The aim of this study was to assess the strength of EBV-specific CTL responses in HD patients, comparing EBV-associated and non-associated cases, relative to healthy individuals. Five of thirteen HD cases and five of eight controls showed regression within the normal range. One EBV-associated HD case displayed regression representative of a CTL response at the weaker end of normal.

Individuals displaying regression within the normal range occurred in both the healthy controls and the HD cases. The mean value for minimum initial cell number giving 50% regression was $1.7x10^5$ for HD cases and $0.8x10^5$ for controls. Among the HD cases, regression was seen in one out of the three EBV-associated cases, refuting the null hypothesis that people who develop EBV-associated HD do not have EBV-specific memory CTLs. However, the minimum initial cell concentration for 50% regression in this individual was $3.3x10^5$ cells/well, which is at the weaker end of the range for normal responses, while the rest of the HD cases were in the range 1-$1.9x10^5$. Thus the possibility remains that there are diminished CTL responses in these cases, relative to other HD cases and to healthy controls. The issue cannot be concluded with certainty from this study due to the small number of cases included, particularly EBV-associated cases.

One of the unexpected results of this study was the relatively high proportion (38%) of controls which showed no regression. The reasons for this are unclear, as the individuals in question were found to be EBV seropositive. As no batch of assays performed at any time was without at least one sample showing regression, there was no reason to doubt the technical aspects of the assays. In the original study by Moss *et al.* (1978), 18 healthy seropositive donors were examined for regression using IVR. All of these showed regression within the normal range, with two of the donors being at the weaker end of the range (50% endpoint = $4x10^5$ cells/well). A further study from this group looked at 22 healthy EBV-positive donors. Variable degrees of regression were seen within the normal range, with two donors exhibiting only weak regression (Moss *et al.*, 1981). However, in a study of NPC patients performed by Chan & Chew (1981), 20 of 38 healthy controls did not exhibit any regression within the limits of their assay. There is no obvious explanation for these differences but it is harder to draw conclusions from a study in which a high proportion of individuals are "non-regressers".

The results of this study negate the suggestion that individuals with EBV-associated HD lack EBV-specific CTLs, but leave open the possibility that these persons have diminished CTL responses to EBV. These data emphasise the need for further investigations into EBV-specific CTL response in HD patients. It is obvious from the results that it would be necessary to analyse samples from a much larger number of HD cases by this method before the significance of potential differences between EBV-associated and non-associated cases could be assessed. However, use of the IVR assay has confirmed that EBV-specific CTLs do exist in the peripheral blood of some cases of EBV-associated HD, an issue which had not been addressed at the start of this work. For future investigation, analysis of specific responses to peptides of individual EBV proteins may prove more fruitful. It is possible that the CTLs are specific for peptides of the immunogenic EBNA-3 family of proteins. Such CTLs, although able to regress LCL-like outgrowths in the IVR which are probably expressing a Lat III phenotype including the EBNA-3s, would not be effective against EBV-positive RS cells *in vivo* which express Lat II, i.e. no EBNA-3 antigens.

This study provides potential evidence of diminished responses to EBV occurring in EBV-associated cases of HD. During the course of the work of this thesis, Dolcetti *et al.* (1995) published a study which looked at T-cells isolated from the lymph node of a single case of non-EBV-associated HD. EBV-specific CTL responses to LMP-2 and, to a lesser extent, EBNA-3c, were observed. The investigators conclude that the inherent immuno-suppression associated with HD does not include diminished memory CTL responses. They also suggest that their findings lend weight to the theory that EBV-associated and non-associated disease are characterised by distinct pathogenic mechanisms.

A second related study went on to examine the CTL responses within the lymph node environment in greater detail (Frisan *et al.*, 1995). This type of study is critical in determining whether the responses in peripheral blood reflect those in the lymph node. Frisan *et al.* investigated samples of T-cells isolated from both blood and lymph node from cases of EBV-associated and non-associated cases of HD. EBV-specific CTLs were found in the lymph nodes of EBV-negative cases but were not detectable in nodes from EBV-positive cases of HD. One of these EBV-positive cases was subjected to investigation of a paired sample of blood and lymph node, and this did have detectable CTLs to EBV present in the peripheral blood even though they were absent from the lymph node CTLs. These data, although preliminary,

support the suggestion that EBV-specific CTL responses may be different between the two groups of HD cases, and that diminished responses in EBV-associated cases may be an important factor in disease pathogenesis. There may be cytokine-mediated suppression of EBV-specific cytotoxicity in the lymph node environment.

Other defects in the effector arm of the immune response in HD remain to be investigated. Absolute numbers of CD8+ CTLs may be reduced in HD. The decreased ratio of CD8+/CD4+ T-cells in the reactive cellular infiltrate in HD is well documented (Poppema & Visser, 1995), and may be a feature of the immunosuppression associated with HD. Alternatively it may be a result of local cytokine production within HD lesions, perhaps even by the RS cells themselves, which selects against the emigration of CD8+ cells into the node.

Chapter 4

Determination of HLA-A2 antigen status in Hodgkin's disease patients and analysis of an HLA-A2-restricted epitope of LMP-2

4.1 Introduction

There is now very good evidence for an association between EBV and a proportion of HD cases. In these cases, viral genomes can be detected in the RS cell population and the clonality of these genomes can be demonstrated (Jarrett *et al.*, 1991). There is a Lat II pattern of expression of viral gene products (Pallesen *et al.*, 1991; Deacon *et al.*, 1993; Grasser *et al.*, 1994), including EBNA-1 and the latent membrane proteins LMP-1 and LMP-2a and -2b.

A high level of LMP-1 protein expression has been demonstrated in RS cells (Pallesen *et al.*, 1991; P.Murray *et al.*, 1992; Armstrong *et al.*, 1992). LMP-2 expression has been demonstrated at the mRNA level but has not yet been confirmed at the protein level, due to the lack of availability of suitable antibodies to the protein. The LMP-2s are presumed to be expressed as protein alongside LMP-1 and, as the two colocalise in patches at the cell membrane (Longnecker & Kieff, 1990), it would be realistic to assume that the level of protein expression of LMP-2 is comparable to that of LMP-1. The LMPs are potentially immunogenic proteins and CTL responses have been demonstrated to peptides of LMP-1 and LMP-2 (Khanna *et al.*, 1991; 1992; R.J.Murray *et al.*, 1988; 1992; Lee *et al.*, 1993). Therefore it may be postulated that presentation of epitopes of these viral proteins in an appropriate HLA class I background may protect against EBV-associated forms of HD.

It is clear that RS cells are not successfully eliminated by the CTL response. There are several possible explanations for this. First, EBV-associated HD may occur only in individuals expressing HLA class I alleles which, by virtue of the architecture of their antigen binding site, are unable to present peptides from the LMPs. Secondly, epitopes eliciting immunodominant CTL responses may have undergone mutation in individuals with HD. Thirdly, HLA class I proteins may not be expressed on the surface of RS cells. Finally, effector responses may be impaired. In these experiments we tested the first two of the hypotheses listed above.

HLA-A2 antigen-positive individuals were the focus of this study, as the HLA-A2 molecule is known to present at least one epitope of the LMP-2 proteins and generate a CTL response (R.J.Murray *et al.*, 1992; Lee *et al.*, 1993). Presentation of this target epitope of LMP-2a occurs in conjunction

with the HLA-A*0201 allele of the A2 family. Possession of the HLA-A2 allele, specifically allele A*0201, may therefore convey a decreased risk of developing EBV-associated HD. To test this hypothesis we studied the frequency of occurrence of the HLA-A2 antigen in EBV-associated and non-associated cases of HD, and compared this to the frequency in the general population. Selected cases which were HLA-A2-positive were investigated further to determine which alleles of HLA-A2 were present.

The possibility of viral escape from CTL surveillance through mutations in target CTL epitopes *in vivo* has been previously described, particularly in the case of genetically unstable viruses, for example with HIV infection of humans (Phillips *et al.*, 1991). There is also an animal model illustrating this phenomenon in the case of lymphocytic choriomeningitis virus (Pircher *et al.*, 1990). It is less well documented for the genetically stable DNA viruses with long histories of host-virus coexistence, however, there are reports of viral escape mutants of EBV occurring in more geographically isolated, healthy populations (de Campos Lima *et al.*, 1993; 1994).

In this study we looked at the possibility of viral escape mutants, possessing a novel sequence of the LMP-2 epitope, occurring in cases of EBV-associated HD arising in an HLA-A2 antigen-positive individual. The epitope which was investigated represents amino acids 426-434 of the LMP-2a protein (Lee *et al.* , 1993).

4.2 Materials & methods

4.2.1 Case and sample selection

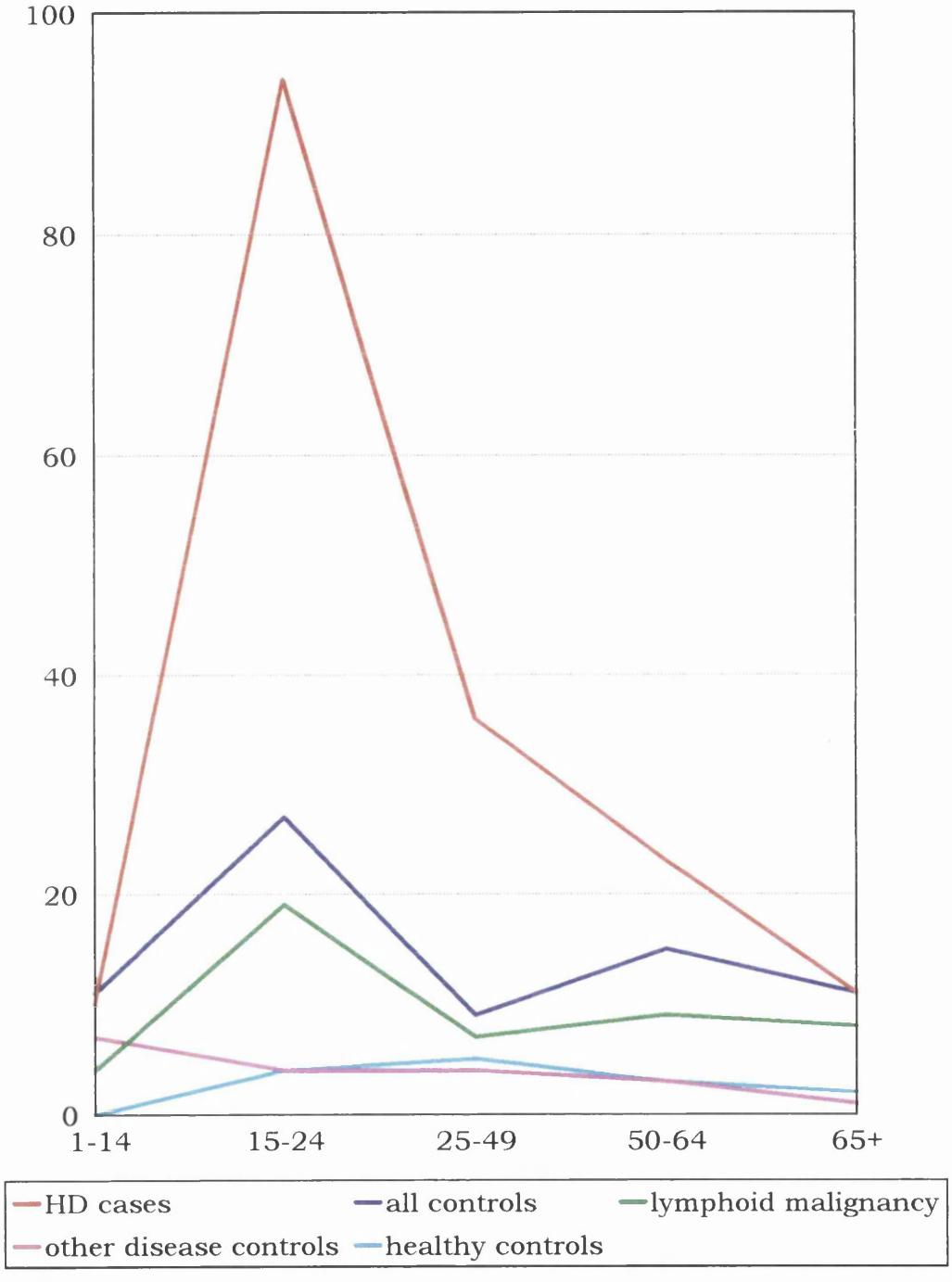
Overall, 410 samples from 313 individuals, including 205 HD cases, were investigated. Samples from 149 individuals were collected as part of a case-control study investigating lymphoid malignancy occurring in young adults aged 15-24 years in England and Wales. Peripheral blood samples were collected from all individuals in this study, and paraffin-embedded biopsy material was obtained from cases. In order to increase the age range of HD cases included, peripheral blood and biopsy samples were collected from 164 cases of lymphoid disease occurring in Scotland and the Northern Region of England. The age distribution of cases and controls is given in Fig.4.1. All cases included were part of population-based studies, thus eliminating potential selection bias. Peripheral blood samples were collected from 15 additional healthy individuals to augment the numbers of controls in the study. HLA-A2 status was determined using PBMCs, LCLs or biopsy material. EBV status was determined using paraffin-embedded biopsy material.

4.2.2 Sample preparation

PBMCs were separated from whole blood using a Ficoll-paque density gradient (section 2.1.1.2). LCLs were established where possible by infection of PBMCs with EBV from the B95-8 cell line, as described in section 2.1.2.3. Cell suspensions were obtained from biopsy samples by manual disruption of the tissue; in some cases frozen biopsy tissue was used as described below.

Cell lysates of PBMC pellets were prepared as described in section 2.1.3.2. DNA was extracted from LCL pellets using proteinase K digestion and organic solvent extraction (section 2.2.1.1). DNA was similarly extracted from fresh or frozen biopsy tissue following initial maceration using a stomacher (Seward Medical Ltd.). DNA was extracted from formalin-fixed, paraffin-embedded tissue sections by digestion in buffer containing 10mM Tris pH 7.4,, 100mM sodium chloride, 25mM EDTA, 0.5% SDS and 200μg/ml proteinase K for 16 hours at 37°C (Shimizu & Burns, 1995), followed by organic solvent extraction (section 2.2.1.1) and isopropanol precipitation (Shimizu & Burns, 1995). All DNA samples were resuspended in nuclease-free water (Pharmacia Biotech).

Fig.4.1 Age distribution of cases and controls



4.2.3 Determination of EBV status

Paraffin-embedded lymph node biopsy sections mounted on APES-coated slides were used for EBER *in situ* hybridisation analysis, as described in section 3.2.3. EBER *in situ* hybridisation was carried out by Alison Armstrong and Diane Gray.

4.2.4 Determination of HLA-A2 antigen status

Three approaches were used to determine HLA-A2 antigen status of cases and controls. First, flow cytometric analysis was used in cases where viable cells or LCLs were available. Secondly, in order to increase the number of cases which could be included in the study, we attempted to develop an immunohistochemical assay to identify the HLA-A2 antigen in formalin-fixed, paraffin-embedded tissue sections, as discussed in section 4.2.4.2. Finally, a PCR strategy was introduced to determine HLA-A2 status using DNA samples and cell lysates prepared from non-viable PBMC pellets.

4.2.4.1 Flow cytometric determination of HLA-A2 status

Flow cytometry was carried out on a total of 163 samples from 158 individuals. Sample types used for this analysis included PBMCs obtained from whole blood (n=19), single cell suspensions from disrupted lymph nodes (n=58) and EBV-immortalised LCLs (n=11). In later experiments whole blood preparations were also used (n=75).

The primary antibody was the BB7.2 monoclonal antibody to HLA-A2, which was in the form of tissue culture supernatant from the hybridoma cell line HB82 (Table II.iii). BB7.2 recognises HLA-A2 and a low frequency variant of HLA-A28 (Parham & Brodsky, 1981). It is an IgG_{2b} class antibody which recognises its substrate via a bivalent interaction (Parham & Brodsky, 1981). An isotype-matched murine monoclonal antibody was used as a negative control in all experiments.

The positive control cells utilised in these experiments were from the VES LCL which is homozygous for HLA-A2. The negative control cells used were from the Jijoye cell line which is HLA-A2 negative. Both VES and HB82 were kindly provided by Dr. P. Chandler.

Approximately $2x10^6$ cells were assayed per sample. All steps were carried out at room temperature unless otherwise stated. Cells were washed twice in 1x PBS containing 0.01% BSA and 0.1% azide (PAB). In order to block any non-specific binding sites, cells were incubated with a solution of 20% normal rabbit serum (SAPU) in PBS for 30 minutes. Following a washing step, cells were split into equal aliquots and incubated with 40µl of either BB7.2 or the control primary antibody for 30 minutes. Following washing in PAB, cells were resuspended in 10µl undiluted goat-anti-mouse FITC-conjugated secondary antibody (Dako Ltd.) for 30 minutes. Finally, cells were washed twice in PAB and fixed in 1% paraformaldehyde.

For whole blood preparations, 200µl of blood were used. Following incubation with secondary antibody, whole blood lysis was performed using the commercially available Immunolyse kit (Coulter Electronics Ltd.) following the manufacturers instructions. This resulted in lysis of red blood cells and therefore reduced non-specific background labelling. The cells were analysed using a Coulter EPICS Elite flow cytometer. EPICS analysis of lymph node cell suspensions was carried out by Linda Andrew.

4.2.4.2 Immunohistochemical determination of HLA-A2 status

Flow cytometry was used exclusively on samples from which viable material was available. In order to increase the number of cases which could be included in the study, we explored the use of an immunohistochemical assay to determine HLA-A2 antigen status. The assay utilised the BB7.2 monoclonal antibody used for flow cytometric analysis. This antibody had been shown to work on cytospins and frozen tissue sections (Poppema & Visser, 1994a) but no data were available regarding its use on paraffin-embedded material. The advent of microwave techniques for unmasking antigens has enabled many antibodies previously only used on frozen tissue or cytospins to be used on formalin-fixed, paraffin-embedded material.

The assay was initially optimised using sections of paraffin-embedded cell pellets of HLA-A2-positive and -negative control cell lines. The basic immunohistochemical techniques used are described in section 2.2.7. Optimisations included comparisons of ABC and APAAP staining methods, variation of antibody incubation times and temperature, and addition of extra blocking agents such as 0.1% BSA to wash buffers. Once the assay was optimised on controls it was tested on clinical samples. Unfortunately a satisfactory signal to noise ratio could not be obtained on these samples, and no further optimisation was attempted.

4.2.4.3 Analysis of HLA-A2 antigen status by PCR

A PCR assay was introduced and used to analyse DNA samples (n=27) and cell lysates (n=198). Each PCR sample was assayed alongside a mock lysate which was handled with the sample from the time of sample preparation. Positive controls for each batch of samples included DNA and cell lysates from the JY and VES cell lines; JY is an LCL which is homozygous for HLA-A*0201. Negative controls for each experiment consisted of DNA and cell lysates from the Jijoye cell line.

PCR primers specific for exons two and three of the HLA-A2 gene were used to amplify a 813bp fragment (Krausa *et al.*, 1995). The primer sequences are detailed in Table IV.i. The reaction conditions were as described in section 2.2.2.1. The optimal concentrations of magnesium chloride and primers included in the reaction were 1.5mM and 0.5μM respectively, as determined by titration (section 2.2.2.2). The PCR buffer used was as detailed in Table II.v; this was different from that used by Krausa *et al.* (1995). A set of internal control primers, also at 0.5μM, were included in the reaction to verify the presence of amplifiable DNA. These were specific for a 330bp fragment of the β_2m gene (Krausa *et al.*, 1993) and the primer sequences are detailed in Table IV.i.

Thermal cycling was performed as described by Krausa *et al.* (1995), using a GeneAmp® PCR system 9600 (Applied Biosystems Ltd.). Cycling parameters were as follows:

Initial denaturation at	95°C for 7 minutes;
Five cycles of	95°C for 1 minutes,
	65°C for 1 minutes,
	72°C for 2 minutes;
Twenty five cycles of	95°C for 1 minutes,
	55°C for 1 minutes,
	72°C for 2 minutes;
Final polymerisation at	72°C for 7 minutes;
Soak at	4°C indefinitely

The 65°C reannealing temperature for the first five cycles was used to reduce non-specific priming (Krausa *et al.*, 1995).

Table IV.i Primer specifications

primer	*nucleotide sequence 5'-3'*	*length*
HLA-A2 5' primer	CCT CGT CCC CAG GCT CT	17
HLA-A2 3' primer	TGG CCC CTG GTA CCC GT	17
β_2m 5' primer	CGA TAT TCC TCA GGT ACT	18
β_2m 3' primer	CAA CTT TCA GCA GCT TAC	18
subtyping primers		
3	GAC GGG GAG ACA CGG AAA	18
22	CAC TCC ATG AGG TAT TTC TA	20
26	GAC GGG GAG ACA CGG AAT	18
27	CAC TCC ATG AGG TAT TTC TT	20
55	AAG GCC CAC TCA CAG ATT G	19
N	CCC CAC GTC GCA GCC AA	17
Q	CTC CAG GTA GGC TCT CAA	18
R	CTC CAG GTA GGC TCT CTG	18
AE	TCC GCC TCA TGG GCC GT	17
AF	ACG TCG CAG CCA TAC ATC A	19
AK	ACT GGT GGT ACC CGC GC	17
BJ	CCG ACC CCA CGT CGC AGG CAC	21
BL	CTC TCT GCT GCT CCG CCT	18
*0201/9 from genomic:		
29	CAG CTC AGA CCA CCA AGC A	19
AL	TGG AAG GTT CCA TCC CCT T	19
LMP-2a 5' primer	GTA TCT ATT TGT TAC TCC TG	20
LMP-2a 3' primer	AGC GTG TTA GAC ATC ACC GT	20

Table IV.ii HLA-A2 subtyping: primer combinations and allele specificities

primers used	*alleles identified*	*size of product*	*reaction*
22, Q	*0201, *0204, *0207, *0209, *0211	715	a
22, AK	*0202	579	b
22, AE	*0203	694	c
22, AF	*0204	540	d
3, AK	*0202, *0205, *0214	409	e
27, AK	*0205, *0208, *0214	579	f
22, BJ	*0207	549	g
26, AK	*0208	408	h
27, Q	*0206, *0210, *0214	715	i
27, N	*0210	546	j
55, Q	*0211	522	k
22, R	*0212, *0213	705	l
22, BL	*0203, *0213	695	m
29, AL	*0209	907	209

PCR products were analysed using 8% polyacrylamide gels (section 2.2.3.1) which were subjected to electrophoresis at 120V for two hours. Gels were initially visualised using ethidium bromide staining and UV light. Subsequently, in order to confirm specificity and increase sensitivity, Southern hybridisation was also carried out. PCR products were transferred onto nylon membrane by electroblotting (section 2.2.4.1) and hybridised to a 30-mer internal ^{32}P-labelled oligonucleotide probe (section 2.2.4.3) specific for exon two of the HLA-A2 gene.

4.2.5 Subtyping of the HLA-A2 locus by PCR

Nested PCR "subtyping" analysis was carried out on selected cases, which were identified in the HLA-A2-specific PCR (section 4.2.4.3), to determine the alleles of HLA-A2 which were present. The subtyping protocol was essentially as described by Krausa *et al.* (1995). Control DNAs for alleles A*0201-*0212 were obtained from Dr. Trevor Jones, UK Transplant Support Service Authority, Bristol. The A*0201 control DNA was from the JY cell line which is homozygous for HLA-A*0201.

Following first round amplification of the 813bp fragment (section 4.2.4.3), nested PCR analysis was performed on 14 cases of EBV-associated HD which were HLA-A2-positive. Thirteen sets of primer combinations (a-m) were used to identify alleles A*0201-*0213. To distinguish A*0201 and *0209, PCR from genomic DNA (reaction 209, Table IV.i) was necessary. Primer sequences are detailed in Table IV.i. Allele specificities of primer combinations are detailed in Table IV.ii. DNA from the first round PCR was diluted 1/1000 before inclusion in the nested reaction.

Cycling conditions for the subtyping reaction, as described by Krausa *et al.* (1995), were as follows:

Initial denaturation at	95°C for 5 minutes;
Five cycles of	95°C for 1 minutes,
	65°C for 1 minutes,
	72°C for 2 minutes;
Ten cycles of	95°C for 1 minutes,
	55°C for 1 minutes,
	72°C for 2 minutes;
Final polymerisation at	72°C for 7 minutes;
Soak at	4°C indefinitely.

4.2.6 Amplification of LMP-2 epitope

Cases for this analysis were selected for EBV positivity of RS cells by EBER *in situ* hybridisation. PCR analysis was performed on DNA extracted from tumour material. Initially DNA was extracted from frozen tissue, however in some instances this could not be obtained and DNA was therefore extracted from paraffin-embedded tissue sections (section 4.2.2).

DNA extracted from the Raji cell line, which is infected by EBV, was used as a positive control. DNA prepared from the J-Jhan cell line, which is EBV-negative, was included as a negative control. As described for the HLA-A2 PCR, each assay contained a positive and negative control for EBV and, in addition, each sample included in the experiment had an individual negative control.

PCR was used to amplify a 248bp fragment of the LMP-2 gene containing the CTL epitope sequence representing codons 426-434 of LMP-2a. The primers used for this amplification are detailed in Table IV.i. UlTma™ DNA polymerase (Applied Biosystems Ltd.) was used as the thermostable polymerase since it has proof-reading ability, i.e. it incorporates 3'-5' exonuclease activity. In order to facilitate more accurate amplification, lower nucleotide concentrations were used than in the standard PCR reaction, and the nucleotide mix contained dTTPs, in place of the standard dUTPs, to facilitate subsequent cloning of PCR products. Primer and magnesium concentrations were optimised by titration (section 2.2.2.2). The reaction conditions were as follows.

DNA, 500ng
dNTPs (dTTP), 40μM
1x UlTma™ reaction buffer
UlTma™ enzyme, 3 units
Magnesium chloride, 0.5mM
Primers, 1μM each.

The PCR was performed as a hot start reaction using Ampliwax® beads (Applied Biosystems Ltd.), as per the UlTma™ specification sheet, with the DNA and enzyme in the upper reaction mix and the other reagents below the wax bead. This prevented truncation of the primers, due to the

exonuclease activity of UlTma™, occurring prior to the start of the reaction. Hot start also increases specificity of amplification by eliminating non-specific annealing of primers to sample DNA at low temperatures. The Ampliwax® beads were melted over the lower reaction mix by heating to 80°C for 10 minutes. The final reaction volume was 100µl.

The PCR cycling conditions were as follows.

Initial denaturation at	95°C for 5 minutes;
Forty cycles of	95°C for 1 minute,
	55°C for 1 minute,
	72°C for 2 minutes;
Final polymerisation at	72°C for 7 minutes;
Soak at	4°C indefinitely.

4.2.7 Nucleotide sequencing of PCR products

Products of the LMP-2 PCR were subjected to electrophoresis using 8% PAGE (section 2.2.3.1) to confirm that a fragment of the correct size had been amplified. Fragments were sequenced either directly from the PCR reaction mix or following cloning into pBluescript vector using the pCR-Script™ cloning kit (Stratagene) as described in section 2.2.5.1. In the case of cloned products, nucleotide sequencing was performed on 3-4 independent clones from each case studied, to ensure that the sequence obtained was representative of the main population present. Sequencing was performed on both strands to minimise artefactual errors. Initially, nucleotide sequencing of clones was performed using a Li-Cor DNA 4000 automated sequencer (section 2.2.5.5). Latterly, direct nucleotide sequencing of PCR products was carried out using an ABI Prism™ 310 automated sequencer (Applied Biosystems Ltd.) as described below. Occasional clones were sequenced commercially by Alta Bioscience, Birmingham University. Sequences obtained were analysed and compared within and between individual samples.

4.2.7.1 Nucleotide sequencing using the ABI Prism™ 310

Direct nucleotide sequencing of PCR products was performed as per the chain termination method, utilising fluorescent dye-labelled dideoxynucleotides. Fragments were purified from PCR reactions using the *High Pure* PCR Product Purification Kit (Boehringer Mannheim UK Ltd.), following the manufacturers instructions. Sequencing reactions were performed using the ABI Prism™ Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems Ltd.), as per the manufacturers instructions, using a Thermal Cycler TC1 (Applied Biosystems Ltd.).

4.2.8 Statistical analysis

For the HLA-A2 analyses, individuals were grouped into several different categories. For examination of the HLA-A2 frequency in control groups (non-HD), these were classified as lymphoid malignancy, other disease controls, and healthy controls. For HLA-A2 analysis of HD cases, these were analysed as a whole group or following separation into "classical" HD and HDLP. Where age distribution of cases is analysed, the age groups 1-14 year, 15-24 year, 25-49 year, 50-64 year, and over 65 year are used.

The analysis of the association between HLA-A2 and HD was performed both with and without adjustment for the effect of age. For these analyses, HD cases were considered first as a whole group, and secondly as EBV-associated (EBV+) and non EBV-associated (EBV-) cases. These groups of HD cases were compared to all controls, all sick controls, and all controls except lymphoid malignancy. The data were also investigated for association between HD subtypes and HLA-A2 positivity.

Statistical analyses were performed by Dr. Freda Alexander. Logistic regression was applied using the statistical package EGRET. Odds ratios and 95% confidence intervals are given.

4.3 Results

The study included 313 individuals, comprising 205 HD cases, 78 other lymphoid disorders and 30 healthy controls. The age distribution of individuals included in each case or control group (section 4.2.8) is illustrated in Fig.4.1.

4.3.1 EBV-association

EBER *in situ* hybridisation was performed on samples from 140 cases, of which 132 were HD. Thirty-five of these were positive and 97 were negative for EBER expression. Overall, 27% of HD cases were EBV-associated. Sixty percent of HD cases in the 0-14 year age group, and 60% of cases over 50 years, were EBV-associated. Although the majority of cases analysed were in the 15-24 year age group, this group displayed only 16% EBV-association, which is consistent with previous findings from our group. To summarise, EBV-associated cases were under-represented compared to the expected number for a non-selected group of HD cases. Age distribution and EBV status of HD cases are given in Table IV.iii.

Table IV.iii Age distribution and EBV-positivity of HD cases

age group	*total cases*	*EBV+*	*(%)*	*EBV-*	*(%)*	*cases excluded**
1-14 year	10	6	67%	3	33%	1
15-24 year	94	15	19%	65	81%	14
25-49 year	36	2	9%	21	91%	13
50-64 year	23	8	57%	6	43%	9
>65 year	11	4	67%	2	33%	5

*Cases excluded from the analysis were those for which EBV status was not available.

4.3.2 HLA-A2

Overall, HLA-A2 status was available for 276 of 313 individuals. Flow cytometry results were available for 158 individuals. Fig.4.2 illustrates histograms obtained from flow cytometric analysis of HLA-A2-negative and -positive samples.

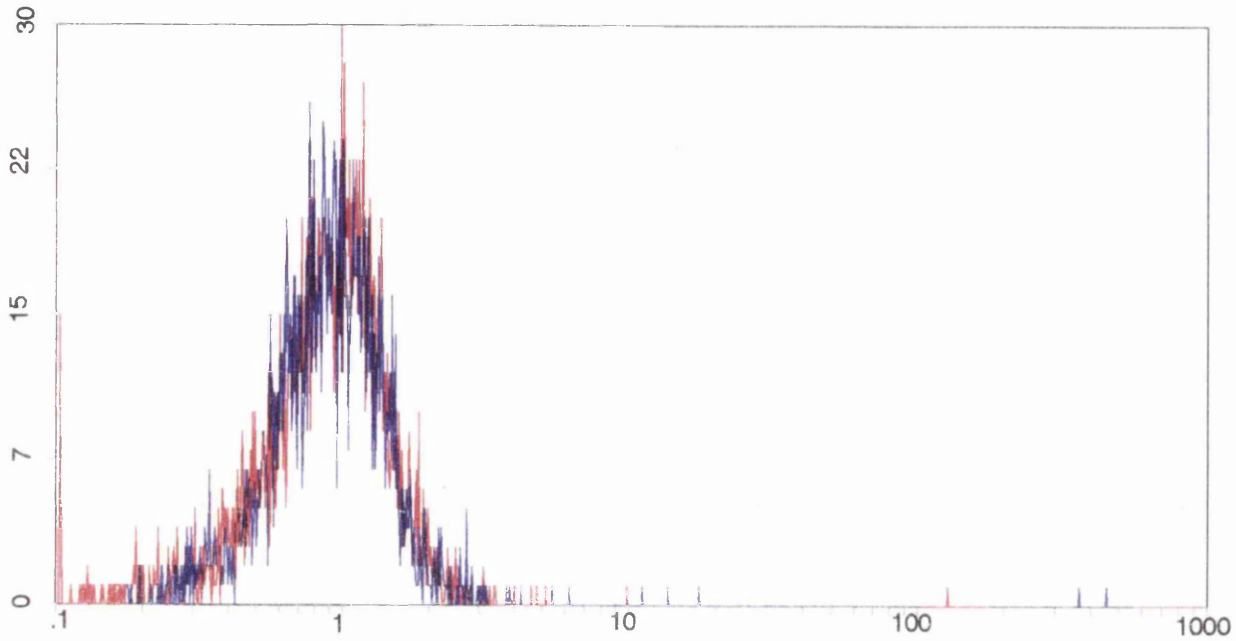
PCR results were available for 187 individuals. A result was only scored if the 330bp product of the β_2m PCR was visible on ethidium bromide-stained gels. Fig.4.3 shows an ethidium bromide-stained polyacrylamide gel of HLA-A2 PCR products.

Both flow cytometry and PCR results were available for the same individual in 68 cases. Of the persons for whom a result was obtained using both assays, only one was discrepant between the two assays, giving a 98.5% correlation. The discrepant case, patient 3090, was A2-negative by flow cytometric analysis but weakly positive by Southern hybridisation following PCR analysis. The β_2m band was strong when visualised with ethidium bromide. It is probable that the band observed on Southern hybridisation was artefactual, and any results for patient 3090 have been omitted from the analyses.

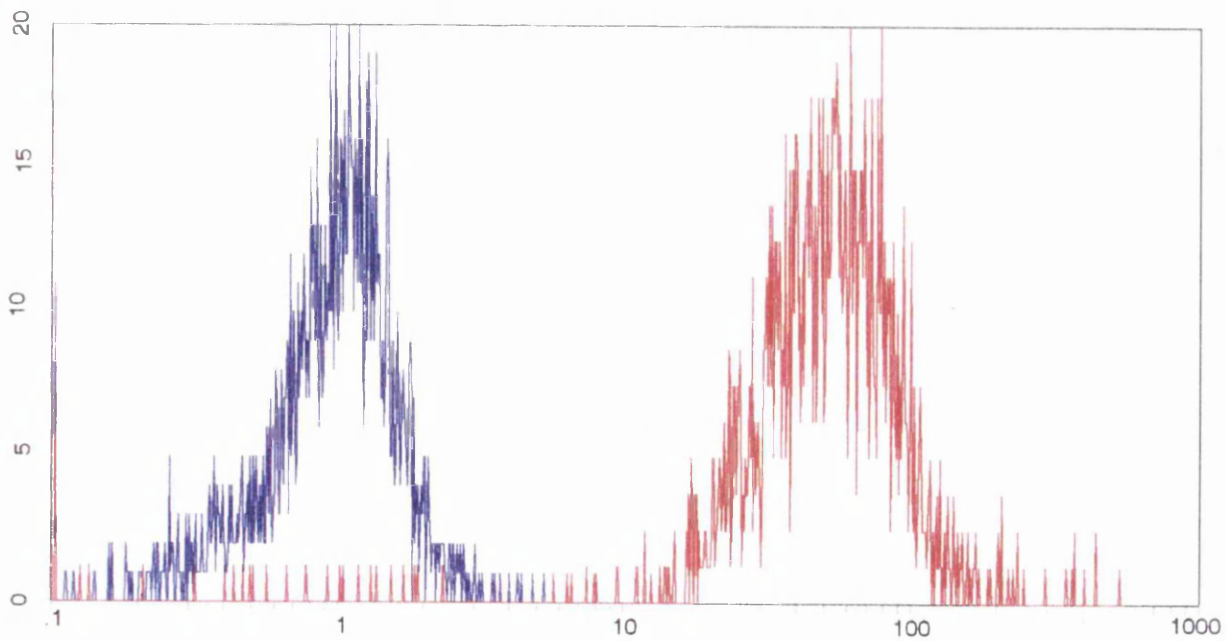
Of the individuals in this study, 122 of 276 (44%) were HLA-A2-positive, which is consistent with the findings of earlier studies and is not significantly different from the expected population frequency of HLA-A2 (Imanishi *et al.*, 1992). HLA-A2-positivity was less than expected for healthy individuals and disease controls (on exclusion of lymphoid malignancy). This may reflect the small numbers of individuals in these two groups. The analysis of HLA-A2 status and HD is illustrated in Table IV.iv. In the unadjusted analysis, there were significant differences between frequencies of HLA-A2 in HD cases and controls. These were 49% for HD cases and 35% for all controls, giving a p-value of 0.025. The difference is not statistically significant following adjustment for age.

Fig.4.2 Flow cytometric analysis of HLA-A2 status

a. HLA-A2-negative sample

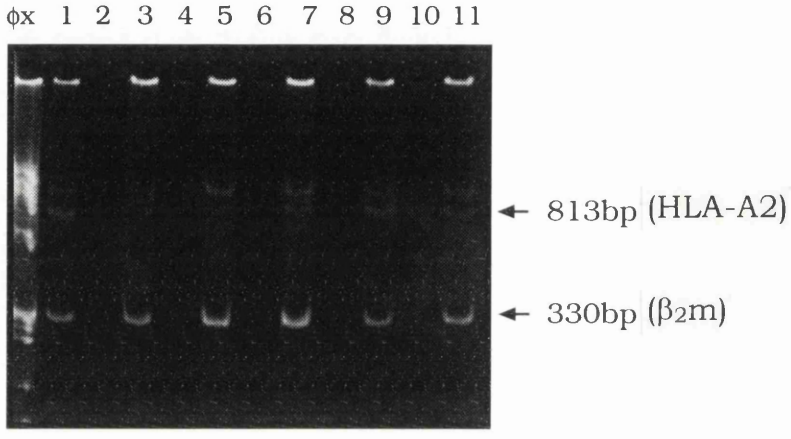


b. HLA-A2-positive sample



NEGATIVE CONTROL
SAMPLE

Fig.4.3 PCR analysis



Lane: φx, size marker; 1, pos control; 2, neg control;
4,6,8,10, water controls; 3,5, HLA-A2-neg samples;
7,9,11, HLA-A2-pos samples

Table IV.iv HLA-A2 and HD

Status	*HLA-A2 +ve*	*Total number*	*% positivity*
all HD cases	86	176	49%
classical HD cases	79	162	49%
all controls	35	100	35%
lymphoid malignancy	24	53	40%
other disease controls	5	20	25%
healthy controls	6	27	22%

Table IV.v HLA-A2 and EBV status

Status	*HLA-A2 +ve*	*Total number*	*% positivity*
all HD cases	86	176	49%
all EBV-associated HD	14	35	40%
non EBV-associated HD	52	97	54%

4.3.3 HLA-A2 and EBV-association

Fourteen of thirty-five (40%) EBV-associated HD cases were HLA-A2-positive, compared to 52 of 97 (54%) of non-EBV-associated cases. Unadjusted analysis of the relationship between EBV status and HLA-A2 antigen positivity is given in Table IV.v. Statistical analysis (section 4.2.8) of this relationship, adjusted for age, is detailed in Table IV.vi. Although fewer of the EBV-associated cases were HLA-A2-positive, the differences observed did not approach statistical significance.

4.3.4 HLA-A2 subtyping

Nested subtyping analysis of the HLA-A2 locus was performed for 14 cases of EBV-associated HD which were HLA-A2-positive by PCR. Thirteen cases typed as HLA-A*0201. One case, patient 2151, typed as HLA-A*0205. Results were obtained for reaction 209 (Table IV.ii), which distinguishes allele A*0201 from A*0209, in six of the thirteen A*0201-positive cases, with these cases typing as A*0201. However, A*0209 is known to be an extremely rare allele in many world populations including Caucasians (M.Browning, personal communication). The results obtained for an A*0201-positive case, and for the A*0205-positive case, are shown in Fig.4.4 alongside the results for JY, the A*0201-positive control (results of reaction 209 not shown).

4.3.5 Analysis of LMP-2 epitope

The nucleotide sequence of a fragment containing the epitope corresponding to amino acids 426-434 of LMP-2a was determined for seven cases of EBV-associated, HLA-A2-positive HD and four HLA-A2-negative cases. The epitope present in cells of the Raji cell line was included in this analysis to exclude the possibility of contamination from the PCR positive control. An example of the results of amplification of an LMP-2 fragment, shown on 8% PAGE stained with ethidium bromide, is illustrated in Fig.4.5.

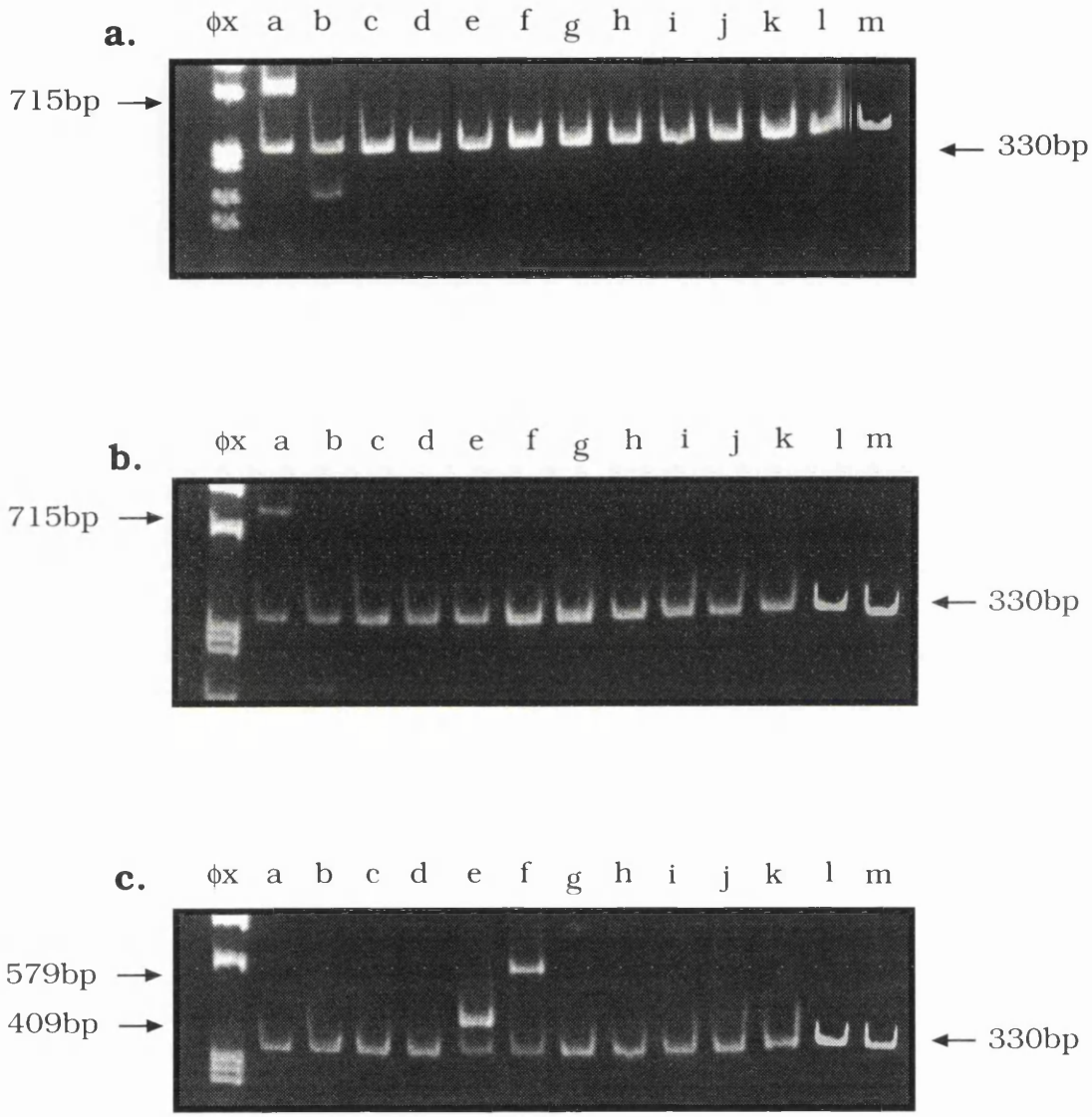
In all of the non-HLA-A2 cases the LMP-2 epitope was conserved. In six of the seven HLA-A2-positive cases studied, the epitope was also conserved. A representative electropherogram from one of these cases is shown in Fig.4.6. In the remaining case, patient 3070, there was a single base-pair change A→T in codon 432. This translates as an amino acid replacement of threonine by a serine residue.

Table IV.vi HLA-A2 status by case/control group

Category of Individual	Proportion HLA-A2 Positive	Odds Ratio[1]	95% Confidence Interval[1]	p-value[1]
HD	86/176	1.00		0.30
All controls	35/100	0.75	0.43-1.30	
HD		1.00		Heterogeneity 0.22 Trend 0.06
Haematological malignancies	24/53	1.07	0.56	
Other hospital controls	5/20	0.46	0.15-1.37	
Healthy controls	6/27	0.41	0.15-1.14	
EBV-associated HD	14/35	1.00		Heterogeneity 0.24 Trend 0.18
Non-EBV-associated HD	52/97	0.84	0.36-1.96	
Haematological malignancies	24/53	1.06	0.50-2.23	
All other controls	11/47	0.45	0.19-1.07	

[1]Adjusted for effect of age.

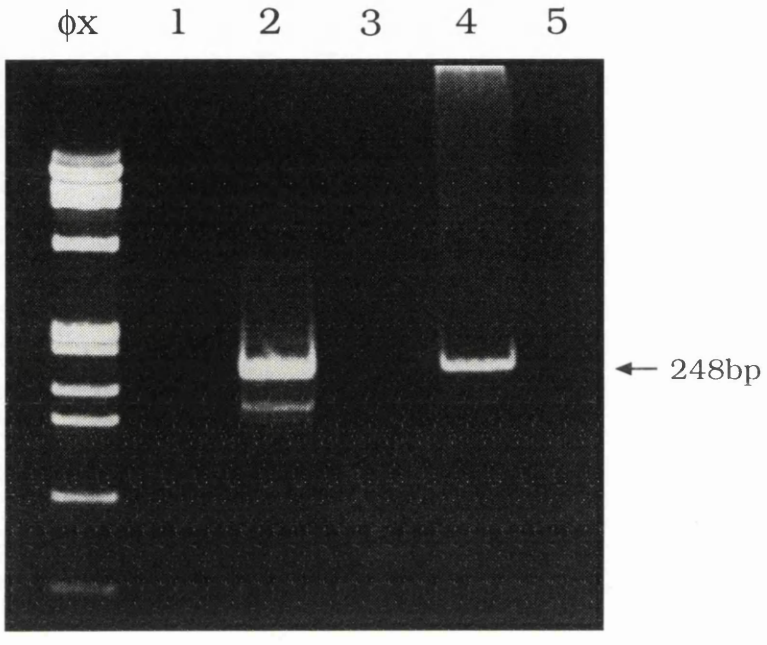
Fig.4.4 Subtyping of HLA-A2 alleles by PCR



a. JY positive control
b. HLA-A*0201-positive sample
c. HLA-A*0205-positive sample

(primer sets a-m detailed in Table IV.ii)

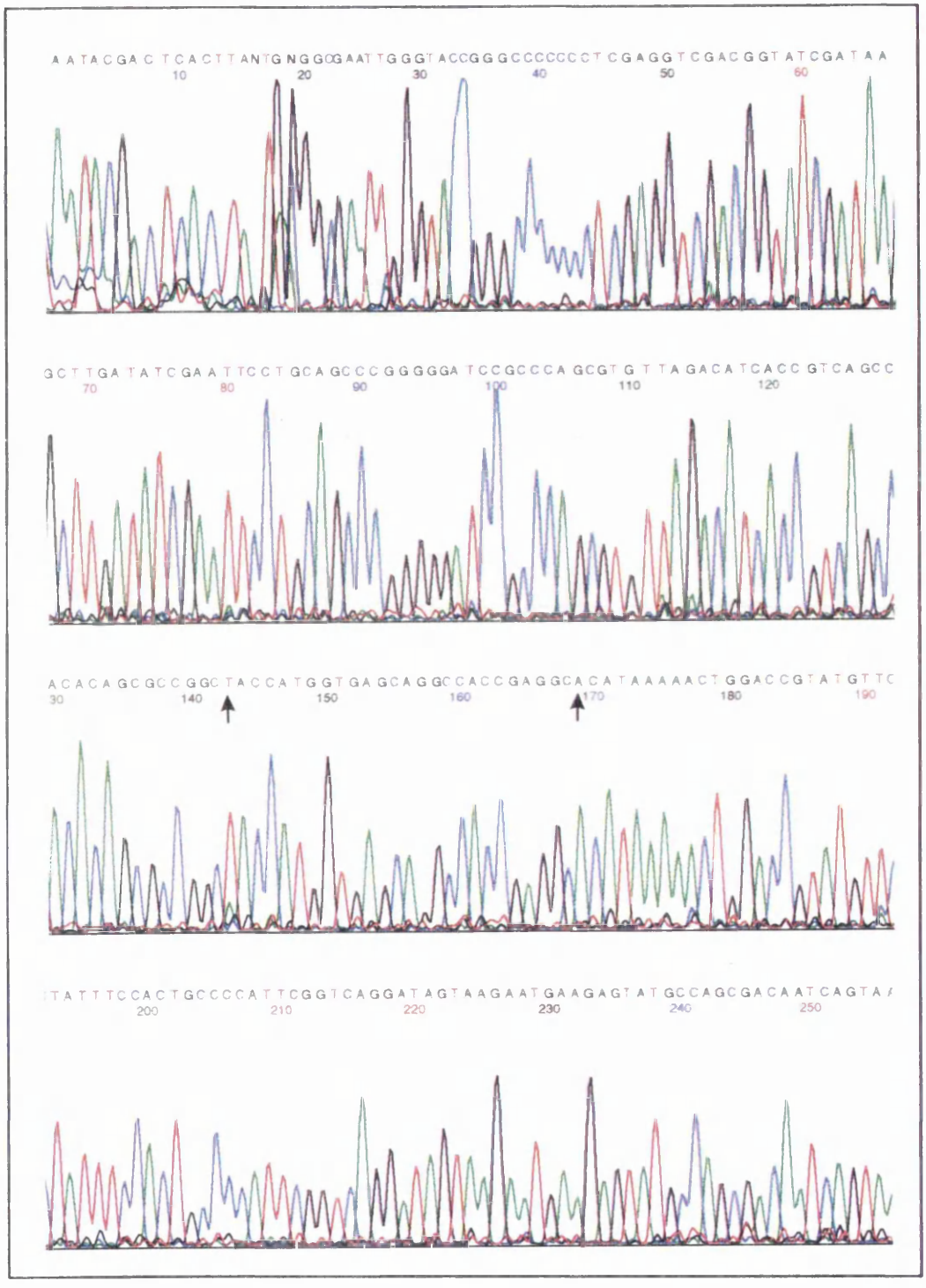
Fig.4.5 8% PAGE showing products of LMP-2 fragment amplification



Lane: φx, size marker
1 neg. control (J-Jhan DNA)
2 pos. control (Raji DNA)
3 blank lane
4 sample 845
5 water control

Fig.4.6 The nucleotide sequence of the 248bp PCR fragment is given above the electropherogram trace. The epitope sequence lies within the arrows.

Fig.4.6 Electropherogram of LMP-2 epitope nucleotide sequencing



4.4 Discussion

In this study we investigated the frequency of occurrence of the HLA-A2 antigen among 313 individuals, including 205 cases of HD. Results were obtained for 276 individuals, of which 176 were HD cases. No significant differences were noted between HD cases and control groups, or between EBV-associated and non-EBV-associated cases of HD. The data from this study therefore argue against the hypothesis that individuals possessing the HLA-A2 antigen are under-represented among cases of EBV-associated HD. These results confirm the findings of a previous study reported by Poppema & Visser (1994a), in which the relationship between HLA-A2 status and HD was investigated in 72 cases. Substantially larger numbers of cases and controls were included in our study, enabling us to confidently corroborate and extend their findings.

In the 1994 study, HLA-A2 status was determined by antibody staining of frozen sections of tumour biopsy material. In our study we extended the analysis to determine which alleles of the A2 family were present. This question has biological relevance since it is known that the CTL epitope representing aa426-434 of EBV LMP-2a is restricted through HLA-A*0201. Ten of the eleven cases of EBV-associated HD which were subtyped at the HLA-A2 locus were found to be A*0201; this allele accounts for 96% of the HLA-A2 alleles in Caucasian populations (Krausa *et al.*, 1995). The remaining case was a young adult Caucasian male, who subtyped as A*0205-positive, an allele more common among African populations (Krausa *et al.*, 1995). The findings of this analysis suggest that the rare alleles of HLA-A2 do not account for the HLA-A2-positive cases of EBV-associated HD.

Seven cases of EBV-associated HD, which were found to be HLA-A*0201-positive, were investigated for the presence of viral escape mutants. A 248bp fragment encompassing the coding sequence of the LMP-2a CTL epitope aa426-434 was amplified and the nucleotide sequence determined. In all but one of these cases the epitope sequence was perfectly conserved with respect to the sequence of the B95-8 viral isolate, as were the epitope sequences of the non-HLA-A2 controls. In one case there was a one base pair change, an A→T substitution at the beginning of codon 432. This results in a threonine to serine change at position 7 of the peptide epitope. As both serine and threonine have uncharged, polar side chains of approximately the same size, and position 7 is not one of the anchor

residues, it is assumed that this will not interfere with the presentation or recognition of the epitope. We conclude from this study that the existence of viral escape mutants at this site is unlikely to be a contributory factor to the pathogenesis of EBV-associated HD. Conservation of this epitope of LMP-2 is clinically relevant as it may be used as a possible target for immunotherapy.

In this study we have shown that possession of the HLA-A2 allele, specifically A*0201, does not protect against development of EBV-associated HD. We have further shown that viral variants, mutated at a key epitope involved in EBV-specific CTL recognition, are not an important feature of the pathogenesis of EBV-associated HD. Other possible mechanisms by which RS cells may survive in the face of the host CTL surveillance have still to be investigated. Some of these are considered below.

There are known to be three CTL epitopes of the LMP-2 protein, however the existence of viral variants with mutations at these sites has yet to be reported. The absence of good monoclonal antibodies to LMP-2 has thwarted efforts to demonstrate the expression of this protein in RS cells. It therefore remains possible that there is a post-transcriptional block in the expression of LMP-2.

LMP-1 is also a potentially immunogenic protein, although responses *in vivo* are rarely immunodominant (Murray *et al.*, 1988; Khanna *et al.*, 1992). Deletion mutants of LMP-1 exist which have decreased immunogenicity in some systems (Khanna *et al.*, 1992; Trivedi *et al.*, 1994). It has been reported that such deleted forms of the protein may lack CTL target epitopes and could therefore contribute to immune evasion (Knecht *et al.*, 1993). However, a more recent study suggests that the existence of accompanying effector cell defects is a necessary criterion for the survival of RS cells expressing such mutant LMP-1 proteins (Santon *et al.*, 1995). The latter study investigated the presence of deleted forms of LMP-1 in HD occurring in both immunocompetent individuals and patients with HIV-related immune suppression. LMP-1 deletion mutants were found more frequently in HIV-associated HD than other HD cases, suggesting that these mutants only persist where immune surveillance is diminished. It seems unlikely, given these observations, that LMP-1 variants contribute significantly to the immunopathology of HD.

Another possible mechanism for immune escape is the lack of adequate presentation of viral antigens at the surface of infected RS cells. It is known that the class I-associated peptide transporter molecules, TAP-1 and TAP-2, are expressed in RS cells (Oudejans *et al.*, 1996; L.S.Young, personal communication). This suggests that the auxiliary machinery for processing and presentation of peptides is intact. There is controversy, however, regarding the ability of class I molecules to reach the RS cell surface. Poppema & Visser (1994b) reported that there was down-regulation of HLA class I expression on the surface of RS cells, however a more recent study found increased HLA class I expression on EBV-positive, as compared to EBV-negative, RS cells (Oudejans *et al.*, 1996). It would seem unlikely therefore, that this is an important mechanism of immune escape in EBV-associated HD.

Due to the nature of RS cells and the lack of *in vitro* systems for the study of HD, it is more difficult to investigate events which are important to disease development and progression within the lymph node environment. There may be local cytokine-mediated suppression of effector cells in the immediate vicinity of the RS cell, or defects in the effector cells themselves; the latter possibility is discussed in chapter 3 of this thesis. Future studies of CTL responses to EBV proteins in blood and lymph node should help resolve this issue.

Chapter 5

Generation and characterisation of B-lymphoblastoid cell lines

5.1 Introduction

LCLs provide a useful renewable viable resource for the future investigation of many aspects of the patient from which they were derived. Throughout the duration of the work described in this thesis, a large number of LCLs, primarily from HD patients, were established. This chapter describes the generation and characterisation of these LCLs.

One of the aims of creating such a resource was to enable a pilot study into genetic instability in HD to be performed. The possibility that abnormal karyotype may be an important feature of HD pathogenesis has been suggested for over 30 years (Spriggs & Boddington, 1962). Since then it has not been extensively studied. Investigations into chromosome banding patterns observed in RS cells of HD report a wide range of cytogenetic features (Teerenhovi *et al.*, 1988). Chromosomal aberrations have been identified in approximately 50% of primary HD cases studied (Banks *et al.*, 1991; Schouten *et al.*, 1989; Tilly *et al.*, 1991) but no consistent cytogenetic markers have been described.

The emphasis of other studies has not been to look for consistent karyotypic markers, but to investigate the separate phenomenon of generalised chromosomal instability as a predisposing factor for development of HD (Barrios *et al.*, 1988; Fonatsch *et al.*, 1989; Kapp *et al.*, 1993). The first two studies examined LCLs and PBMCs from HD patients for abnormalities, rather than attempting to look in RS cells. Twice as many aberrations were observed in PBMCs from untreated HD patients compared to PBMCs from healthy individuals (Barrios *et al.*, 1988). LCLs from HD patients were reported to be more susceptible to drug-induced gross chromosomal mutation than were LCLs derived from healthy donors (Fonatsch *et al.*, 1989). The 1993 study observed a high frequency of karyotypic abnormalities in LCLs arising from HD material serially transplanted into SCID mice (Kapp *et al.*, 1993). These may have arisen from RS cells or bystander B-cells with inherent cytogenetic instability.

In light of these data, six LCLs from HD patients were used in this small pilot study to investigate the occurrence of random chromosomal abnormalities, aiming to assess whether inherent genomic instability, rather than particular cytogenetic markers, is indeed a feature of individuals who go on to develop HD.

5.2 Materials & methods

In most instances, LCLs were established by EBV-infection of PBMCs. Occasionally LCLs arose spontaneously from cultures of cells obtained from lymph node biopsies during work being undertaken concurrently in the unit. Some of these were included in the eventual bank of HD LCLs.

5.2.1 Generation of LCLs

PBMCs were separated using a Ficoll-paque gradient (section 2.1.1.3) and infected with concentrated B95-8 EBV stock (section 2.1.2.1 - 2.1.2.3). Cells were seeded at a density of $2x10^6$/well in 96-well microtitre plates and initially treated with Cyclosporin A at a concentration of 0.1μg/ml to suppress CTL function. Cultures were fed weekly by 50% medium replacement. Evidence of transformation was routinely seen at 10-14 days post infection. Occasionally transformation was not evident until 6-8 weeks post infection, and in these cases it was assumed that the B95-8 virus infection had failed and a spontaneous transformation event, involving latent virus present in the donor PBMCs, had occurred.

After the first signs of transformation were seen, cells continued to be fed weekly by 50% medium replacement for a further 2-3 weeks or until growth was exponential; the cultures were then expanded up to 250ml in six $250cm^3$ tissue culture flasks by splitting 1:2 twice weekly. Viable stocks were stored in liquid nitrogen (section 2.1.1.5) and cells were grown up and used for further analysis as described below.

5.2.2 Characterisation of LCLs

Putative LCLs were characterised for three main reasons. First, to determine whether they were indeed a B-cell line. Secondly, to confirm the presence of EBV and determine whether they expressed viral latent antigens. It was desirable to know if the virus was B95-8 or originated from the patient's latent virus reservoir. Thirdly, LCLs were investigated to confirm their individuality, i.e. that they were indeed derived from the patient's PBMCs.

Fixed cells were taken and phenotyped using flow cytometry. Cell lysates were prepared and used in Western blot EBNA-2 typing to establish the identity of the virus present. LCLs were examined immunohistochemically to determine viral antigen expression. Forensic typing was performed on DNA extracted from LCL pellets.

5.2.2.1 Phenotyping of LCLs by flow cytometry

A panel of FITC-conjugated antibodies was used to phenotype putative LCLs (Table V.i). The method used was essentially as described in section 4.2.4.1, except that this was a direct labelling technique. Flow cytometric analysis was carried out using an EPICS Elite (Coulter Electronics Ltd.).

Table V.i Antibodies used in flow cytometry

antibody to	*expression pattern*
CD2	all T-cells
CD23	activated cells, e.g. EBV-infected
CD45	haemopoeitic cells
CD14	myeloid cells - monocytes
CD19	all B-cells
CD30	activated cells, LCLs
EMA	epithelial cells
CD15	granulocytes, activated cells
κ/λ	B-cells (express one or other)

5.2.2.2 EBNA-2 typing by Western blot analysis

The basic method used is as described in section 2.2.6. Several optimisations were made, which included the following: nuclear enrichment lysis was performed, using lysis buffer (Table II.viii) containing only 0.1% Triton X as detergent, which disrupted the plasma membrane only, allowing the nuclei to be purified by pelleting at 13,000rpm in an MSE microfuge for 10 minutes at 4°C. The pellet of nuclei was then subjected to lysis in standard buffer (Table II.viii). Western blots were performed using a B95-8 cell lysate as a control. Lysates prepared from an uninfected J-Jhan cell line and a mouse fibroblast line were included as negative controls.

A second modification consisted of the addition of 20% heat-inactivated normal sheep serum (SAPU) to all Marvel buffer, prior to incubation with secondary antibody. The secondary antibody used in this procedure was raised in sheep, therefore this blocked any non-specific binding sites present on the membrane and reduced background signal.

5.2.2.3 Immunohistochemical Analysis

Immunohistochemical analysis was carried out on cytospins of the putative LCLs to investigate expression of EBV antigens. Cytospins of each LCL were prepared (section 2.1.3.1) and stained for EBNA-2 and LMP-1 using APAAP and ABC staining methods respectively (section 2.2.7). For EBNA-2 staining, PE2 primary antibody was used and for detection of LMP-1, CS1-4 was the antibody of choice. Antibody to rotavirus antigens was used as a negative control antibody. Cytospins of a previously characterised LCL (not described here) were included in each assay as a positive control, and cytospins prepared from the uninfected J-Jhan cell line were used alongside these as a negative control.

5.2.2.4 D1S80 forensic typing of LCLs

At certain time points in this study, large numbers of LCL cultures were being generated and handled concurrently. It was necessary to confirm individuality of LCLs before further analyses were carried out.

The human genome contains a large number of repetitive DNA sequences, arranged as tandem repeat units, which are known as variable number of tandem repeats, or VNTRs (Nakamura *et al.*, 1987; 1988). Individuals possess different numbers of repeat units, therefore VNTRs are classed as "length" polymorphisms rather than sequence polymorphisms. The VNTR marker D1S80 contains a variable number of 16bp repeat units (Kasai *et al.*, 1990) and can occur as over 400 different genotypes.

Using the commercially available D1S80 forensic typing kit (Applied Biosystems Ltd.) it was possible to distinguish LCLs arising from different individuals. The kit utilised a PCR strategy which exploited the allelic patterns unique to each individual, with a population-based discrimination power of 95-98%.

For D1S80 analysis, $1x10^7$ cells were pelleted, washed twice in sterile PBS, and pelleted at 500*g* for 10 minutes. Pellets were stored at -80°C and subsequently defrosted, protease digested and the DNA recovered using organic solvent extraction (section 2.2.1.1).

PCR reactions were set up as described in the manufacturers instructions. The reaction mix contained reaction buffer, deoxynucleoside triphosphates and Amplitaq® DNA polymerase. Magnesium chloride was added to a final concentration of 1.0mM. Twentyfive nanograms of sample DNA were added to the reaction mix. Positive control DNA, with a known allelic pattern, was included in the kit and run alongside each batch of samples. Water was also included as a negative control. The PCR reaction was performed using a GeneAmp® PCR system 9600 machine (Applied Biosystems Ltd.) with thermocycling parameters as follows: Initial denaturation at 95°C for 5 minutes; thirty cycles of 95°C for 15s, 66°C for 15s, 72°C for 40s; final polymerisation at 72°C for 10 minutes. Reaction products were analysed by 8% PAGE (section 2.2.3.1).

5.2.3 Karyotypic analysis

LCLs established from HD patients were sent in culture to Prof. Lorna Secker-Walker, Royal Free Hospital, London, where they were examined for the presence of karyotypic abnormalities. A sample of 10-20 metaphases from each LCL was subjected to cytogenetic analysis.

5.3 Results

Thirty LCLs were generated during the course of this work, from both HD patients and healthy individuals. The first 12 established were extensively characterised and the results are detailed below.

5.3.1 Characterisation of LCLs

LCLs phenotyped included lines which had been in culture for only five weeks and those which had been cultured for more than six months. All LCLs analysed were polyclonal populations of B-cell origin as demonstrated by flow cytometry; the longer the LCL had been in culture the larger the main clonal population. CD23, CD30 and CD45 were expressed by all LCLs.

Of the 12 LCLs which were analysed by EBNA-2 typing, seven appeared to contain B95-8 virus. The remaining five contained an EBNA-2 species which had a slightly different electrophoretic mobility to the B95-8 protein, most of which were smaller.

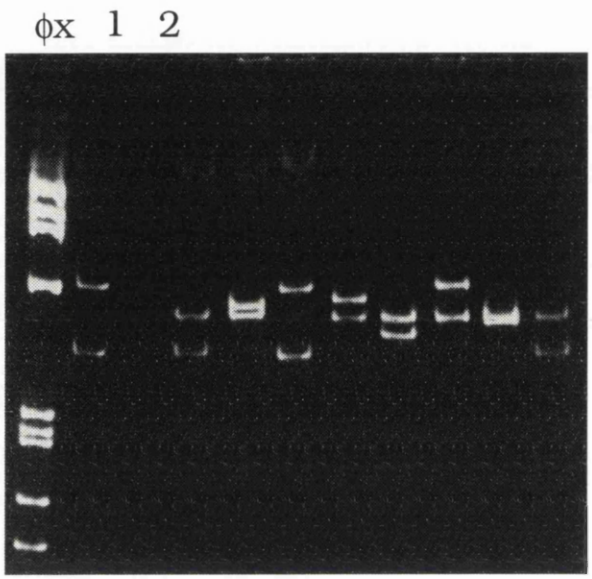
Immunohistochemistry was successfully performed on eight LCLs, all of which were LMP-1 positive. In six cases EBNA-2 expression was clearly demonstrable. In the remaining two cases the staining was faint and uninterpretable.

Forensic typing by PCR revealed that all LCLs were indeed unique lines derived from separate individuals (see Fig.5.1).

5.3.2 Karyotypic analysis

Six LCLs established from HD patients were analysed. Three of these were karyotypically normal in all metaphases examined. Results for the remaining three LCLs are described in Table V.ii.

Fig.5.1 8% PAGE showing results of forensic typing PCR analysis



Lane: ϕx, size marker;
1, pos control;
2, water control;
selection of LCLs

Table V.ii Cytogenetic results

LCL	*no. cells*	*results*	*analysis*
2459	10	46,XY; add (22) (q13)	extra material on chr.22 - not of chr.22 origin - not balanced translocation
2686	(20) 18	46,XY; normal	
	1	45,XY; missing chr.2	random chromosome loss;
	1	45,XY; missing chr.13	no chromosome breakage
2802	(20) 10	46,XY; normal	
	10	46,XY; der(6) t(5;6)	

5.4 Conclusions

Thirty LCLs were stored during the course of this work. They will provide a useful resource for future genetic analyses and will also be available for further immunological studies of CTL responses in HD.

A subset of these LCLs were karyotyped in order to investigate whether genetic instability is a feature of HD as previously suggested. Three of the six lines had abnormalities and therefore the results were somewhat inconclusive. The findings do suggest that this may be a worthwhile area for further research. Future studies, however, must involve larger case numbers alongside LCLs from a comparable number of healthy control individuals, and allow for comparisons of passage number.

Chapter 6

General Discussion

One of the most notable advances in the study of HD in the past 25 years has been to link the disease to a viral agent, specifically to associate a subset of HD cases with the herpesvirus EBV. This answered several questions relating to the aetiology of these disease cases, but posed many others. Although the bank of evidence supporting the association is now large and widely accepted, the exact role played by the virus in EBV-associated HD is still not understood.

In the proportion of HD cases which are EBV-associated, around 40% in developed countries, latent virus is detectable in the tumour cells, the RS cells. These cells express viral latent antigens EBNA-1, LMP-1 and LMP-2 (Pallesen *et al.*, 1991; Deacon *et al.*, 1993; Grasser *et al.*, 1994). The persistence of RS cells in HD lesions, without recognition or elimination by circulating CTLs, is an enigma in EBV-associated cases in which immunogenic viral membrane proteins are expressed. How do EBV-positive RS cells survive in the lymph node in the face of the CTL immune response? Several theories concerning this issue were addressed during the course of the work for this thesis.

HLA class I-restricted CTL responses to peptides of the LMPs have been demonstrated *in vitro* (R.J.Murray *et al.*, 1988; 1992; Khanna *et al.*, 1992; Lee *et al.*, 1993). Thus it was postulated that EBV-associated HD would not occur in individuals expressing HLA class I alleles which present epitopes from the LMPs. HLA-A2 was the allele investigated in our study, as it is known to present an epitope of LMP-2 and generate a CTL response (Lee *et al.*, 1993). The results of the study refuted the hypothesis that HLA-A2 individuals would be under-represented among EBV-associated HD cases.

It is known that there are several alleles in the HLA-A2 family. A*0201 is the commonest in Caucasian populations in which it accounts for 96% of all HLA-A2 alleles (Krausa *et al.*, 1995). It is this allele that presents the CTL epitope representing aa426-434 of LMP-2a. We therefore went on to determine whether rarer alleles of the A2 family account for the observed HLA-A2 antigen-positivity seen in EBV-associated HD. This hypothesis was also refuted.

In light of recent reports of the occurrence of EBV viral escape mutants arising in more geographically isolated populations (De Campos-Lima *et al.*, 1993; 1994), it was thought that such viral variants may contribute to the pathogenesis of EBV-associated HD. Cases of HLA-A2-positive, EBV-

associated HD were investigated for the presence of mutations in LMP-2 which would abolish CTL recognition of an A*0201-restricted epitope. No such mutations were found, although one conservative mutation was identified in one case from an HLA-A*0205-positive individual. In conclusion, it is clear that there are other mechanisms by which RS cells of EBV-associated HD evade immune detection.

An alternative hypothesis was that of RS cells being unable to process and/or present viral antigens to circulating CTLs. However, the cellular mechanism for antigen processing has been found to be intact in these cells (Oudejans *et al.*, 1996; L.S.Young, personal communication). The capacity of RS cells to present peptides of viral antigens at the cell surface remained controversial. In 1994 it was reported that class I molecules were absent from the surface of RS cells in some cases (Poppema & Visser, 1994b). Since then this finding has been contradicted, with a report of class I expression being present on RS cells of both EBV-associated and non-associated HD (Oudejans *et al.*, 1996).

It has long been known that immune dysfunction accompanies the development of HD (Kadin, 1992), although there is still debate regarding how important this is in disease development. We performed a series of *in vitro* assays to examine the EBV-specific CTL responses in HD patients relative to healthy people, to determine whether a decrease in these CTL responses was a feature of HD. In addition, we compared EBV-associated and non-associated HD cases to establish whether decreased EBV-specific responses may predispose to EBV-associated HD, or whether all HD patients had decreased responses due to the immunosuppressive nature of the disease.

The results of our study found slightly lower levels of EBV-specific CTL responses in HD patients relative to healthy individuals. We also observed diminished responses in EBV-associated HD cases compared to non-EBV-associated HD, although the numbers of individuals included in the study were small, therefore the results are not statistically significant. We may suggest from our findings that reduced effectiveness of EBV-specific CTLs is a feature of HD, and may be most marked in EBV-associated cases, suggesting this may be a significant factor in the pathogenesis of EBV-associated disease.

The finding that EBV-specific CTL responses do exist in some cases of EBV-associated HD opens up several avenues for the treatment of EBV-associated disease. If the CTL responses present in these patients are directed to the LMPs, then potential immunotherapeutic approaches may be devised to boost these responses in an attempt to achieve effective CTL recognition and elimination of EBV-positive RS cells. This finding also raises the question, however, of why the existing CTL response is failing to do just that. Adoptive transfer strategies may be an effective approach for treatment of HD.

The results of the studies in chapter four of this thesis confirm that at least one CTL epitope of LMP-2 is present and not mutated in HD patients, making this a potential target for immunotherapeutic intervention. Future studies will focus on investigation of the nature of other specific responses present in EBV-associated HD, both in peripheral blood and in the lymph node, which may shed light on other potential targets for adoptive immunotherapy.

One of the confounding features of the study of HD is the absence of suitable *in vitro* systems for the direct study of RS cells. It is probable that RS cells need to be present in a specific cellular microenvironment in order to survive, at least in the early stages of disease development, and this may explain why RS cell lines are difficult to establish in culture. The necessary cellular environment may be provided by the large reactive cellular infiltrate present in HD lesions. There is no suitable system for investigating events which are important to disease onset and development within the lymph node itself. It has been suggested that there may be local suppression of CTL function within the lymph node environment, probably mediated by cytokine expression (Frisan *et al.*, 1995). There is also an observed increase in CD4+/CD8+ T-cell ratios in the reactive cellular infiltrate found in HD lesions (Poppema & Visser, 1995). This may be a reflection of the lack of CD8+ CTLs in these nodes. CD8+ CTLs which can be seen in HD nodes tend not to be in the immediate vicinity of the RS cells, rather the RS cells are rosetted by CD4+ T-lymphocytes. This may also be a function of a particular pattern of cytokine expression by RS cells. Until the RS cells can be successfully separated from the rest of the cellular infiltrate this question will be difficult to address.

The role played by such factors in the pathogenesis of HD remains to be resolved. The main areas which remain to be clarified in future work include determination of the lineage of RS cells and their precursors. Use of cell culture systems, such as those utilising CD40 (O'Grady *et al.*, 1994), may facilitate future *in vitro* propagation of RS cells. Single cell studies and gene expression profiles may shed light on the cytokines expressed by RS cells and cells of the surrounding infiltrate.

In young adult HD cases, which have the strongest epidemiological evidence for an infectious aetiology, an infectious agent has yet to be isolated. When and if this happens, it will shed considerable light on the pathogenesis of these cases, and will provide interesting comparisons with EBV-associated disease.

Appendices

Appendix A: Sources of commonly used cell lines

cell line	*source*
B95-8	Q.Y.Yao, CRC laboratories, University of Birmingham
HB82	P.Chandler, Clinical Research Centre, Northwick Park Hosp., London
Jijoye	ATCC
J-Jhan	M.Steele, Western General Hosp., Edinburgh
Raji	ATCC
VES	P.Chandler, Clinical Research Centre, Northwick Park Hosp., London

Appendix B: List of suppliers

supplier	*location*
A. & J. Beveridge Ltd.	Glasgow
Alpha Laboratories Ltd.	Eastleigh, Hampshire
Amersham International PLC	Aylesbury, Buckinghamshire
Anachem	Luton, Bedfordshire
Applied Biosystems Ltd.	Warrington, Cheshire
BDH (Merck Ltd.)	Lutterworth, Leicestershire
Beckman Instruments (UK) Ltd.	High Wycombe, Buckinghamshire
Bio-Rad Laboratories	Hemel Hempstead, Hertfordshire
Boehringer Mannheim UK Ltd.	Lewes, East Sussex
Costar UK Ltd.	High Wycombe, Buckinghamshire
Coulter Electronics Ltd.	Luton, Bedfordshire
Cruachem	Bearsden, Glasgow
Dako Ltd.	High Wycombe, Buckinghamshire
Epicentre Technologies (Cambio Ltd.)	Cambridge
Fisons Ltd.	Loughborough, Leicestershire
Flowgen Instruments Ltd.	Sittingbourne, Kent
Genetic Research Instrumentation Ltd.	Felsted, Dunmow, Essex
Greiner Labortechnik Ltd.	Stonehouse, Gloucestershire
Gull Laboratories Inc.	Salt Lake City, Utah, USA
ICN Biomedicals Ltd.	High Wycombe, Buckinghamshire
I.G.I. Ltd	Sunderland, Tyne & Wear
Leo Laboratories	Princes Risborough, Buckinghamshire
Li-Cor	Lincoln, NE, USA
Life Technologies Ltd.	Inchinnan, Renfrewshire
Millipore (UK) Ltd.	Watford, Hertfordshire
National Diagnostics	Atlanta, Georgia, USA
Pharmacia Biotech	Knowhill, Milton Keynes
Promega UK Ltd.	Chilworth, Southampton
Qiagen Ltd.	Crawley, West Sussex
Rathburn Chemicals Ltd.	Walkerburn, Scotland
Sandoz Pharmaceuticals	Camberley, Surrey
SAPU	Carluke, Lanarkshire
Sarstedt Ltd.	Beaumont Leys, Leicestershire
Savant Instruments Inc.	Farmingdale, New York, USA
Scotlab	Coatbridge, Lanarkshire
Seward Medical Ltd.	London
Shandon	Basingstoke, Hampshire
Sigma Chemical Company	Poole, Dorset
Stratagene	Cambridge

Appendix C: Calculation of 50% endpoints using the method of Reed & Meunch

This method of calculating 50% endpoints is used when there is a wide range of variations in titre and is designed to take advantage of results from all dilutions tested, rather than just those around the endpoint. This is achieved by calculating the accumulated number of wells showing regression at and above each dilution and expressing this as a percentage. When using this method of calculation, the assumption is made that a well of culture displaying regression at a low cell concentration would also do so at a higher cell concentration.

The following formula is used for the initial calculation of the percentage of regression:

$$\% \text{ regression at dilution N} = \frac{\text{total no. regressed wells up to and including N}}{\text{total no. wells up to and including N}}$$

In practice the endpoint usually lies between two dilutions. To calculate the actual endpoint it is therefore necessary to determine the proportional distance (D) from the dilution below or above the 50% endpoint. This is achieved using the formula

$$D = \frac{50 - Y}{X - Y}$$

where X = % regression occurring at the dilution above the 50% endpoint
Y = % regression occurring at the dilution below the 50% endpoint

Since cell dilutions are increasing on a logarithmic scale, the final value is calculated as follows:

$$\log(\text{endpoint}) = \log Y + [\log(\text{dilution factor}) \times D]$$

Tabulations used to score IVR assays

*P.no.	cell no.	tfm'n	regr'n	total tfm'n	total regr'n	% regr'n
3248	$6x10^5$	0	6	0	18	100
	$3x10^5$	0	6	0	12	100
	$1.5x10^5$	1	5	1	6	86
	$7.5x10^4$	5	1	6	1	14
	$3.8x10^4$	6	0	12	0	0
	$1.9x10^4$	-	-	-	-	-
3250	$6x10^5$	0	4	0	12	100
	$3x10^5$	0	4	0	8	100
	$1.5x10^5$	1	3	1	4	80
	$7.5x10^4$	3	1	4	1	20
	$3.8x10^4$	4	0	8	0	0
	$1.9x10^4$	-	-	-	-	-
3258	$6x10^5$	-	-	-	-	-
	$3x10^5$	0	6	0	11	100
	$1.5x10^5$	2	4	2	5	71
	$7.5x10^4$	5	1	7	1	13
	$3.8x10^4$	6	0	13	0	0
	$1.9x10^4$	6	0	19	0	0
3277	$6x10^5$	1	5	1	9	90
	$3x10^5$	4	2	5	4	44
	$1.5x10^5$	4	2	9	2	18
	$7.5x10^4$	6	0	15	0	0
	$3.8x10^4$	-	-	-	-	-
	$1.9x10^4$	-	-	-	-	-
3292	$6x10^5$	-	-	-	-	-
	$3x10^5$	0	6	0	13	100
	$1.5x10^5$	0	6	0	7	100
	$7.5x10^4$	5	1	5	1	16
	$3.8x10^4$	6	0	11	0	0
	$1.9x10^4$	6	0	17	0	0

P.no.	*dilution*	*tfm'n*	*regr'n*	*total tfm'n*	*total regr'n*	*% regr'n*
3788	$6x10^5$	0	6	0	18	100
	$3x10^5$	0	6	0	12	100
	$1.5x10^5$	0	6	0	6	100
	$7.5x10^4$	6	0	6	0	0
	$3.8x10^4$	6	0	12	0	0
	$1.9x10^4$	6	0	18	0	0
3888	$6x10^5$	1	5	1	13	93
	$3x10^5$	0	6	1	18	89
	$1.5x10^5$	4	2	5	2	29
	$7.5x10^4$	6	0	11	0	0
	$3.8x10^4$	6	0	17	0	0
	$1.9x10^4$	6	0	23	0	0
3893	$6x10^5$	0	6	0	20	100
	$3x10^5$	0	6	0	14	100
	$1.5x10^5$	1	5	1	8	89
	$7.5x10^4$	4	2	5	3	38
	$3.8x10^4$	5	1	10	1	9
	$1.9x10^4$	6	0	16	0	0
3894	$6x10^5$	0	6	0	20	100
	$3x10^5$	0	6	0	14	100
	$1.5x10^5$	0	6	0	8	100
	$7.5x10^4$	4	2	4	2	33
	$3.8x10^4$	6	0	10	0	0
	$1.9x10^4$	6	0	16	0	0

*P.no., patient number; tfm'n, transformation; regr'n, regression

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