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No correlation between LMP-1 and bcl-2 expression was demonstrated.

No significant difference in Apoptotic Index between LMP-1 (+ve or -ve); Bcl-2 (+ve or -ve) or histological subtypes could be demonstrated.

- 2) A retrospective epidemiological study of patients presenting to these hospitals with a diagnosis of Hodgkin's disease, over the 5 year time period was also conducted (n=175). This included patients in whom archival tissue was not available. These were analysed according to age, race, sex and histological subtype using data from relevant patient files.

Black, White and Asian children (≤ 15 years of age) showed a similar average age of presentation (average: 9yr 7m). Only the Black paediatric group showed the expected increased male:female ratio. Mixed cellularity subtype was commonest in the Black paediatric group, while White paediatric cases were predominantly nodular sclerosis subtype. In Asian children 3/5 and 2/5 cases were mixed cellularity and nodular sclerosis subtype respectively.

Both Black and White adults presented predominantly with nodular sclerosis Hodgkin's disease. Both groups showed a male:female ratio of ~ 1:1. White adults presented at a slightly older average age (41yr 6m vs 32yr 7m). One Asian adult case was of nodular sclerosis subtype.

ABSTRACT

The association between Epstein-Barr Virus (EBV) and Hodgkin's disease, has prompted numerous studies over the last decade. Earlier reports from Europe and North America indicated a prevalence of EBV positive Hodgkin's disease of around 40-50%, especially with mixed cellularity subtype. Recently the prevalence of EBV in Hodgkin's disease in developing countries was found to be higher. No data had been published from South Africa, where, for historical reasons distinct racial communities exist.

- 1) The prevalence of EBV in Hodgkin's disease in 61 paediatric and adult patients at the Baragwanath and Johannesburg Hospitals over a 5 year period (1989-1993), was investigated using immunohistochemistry and *in situ* hybridisation.

Immunohistochemistry for bcl-2 was also performed since upregulation of bcl-2 by EBV Latent Membrane Protein (LMP-1) had previously been postulated as EBV's pathogenetic role in Hodgkin's disease. These findings were then related to Apoptotic Index, using morphological assessment, and *in-situ* end labelling of fragmented DNA.

We found an overall prevalence of EBV positive Hodgkin's disease of 52%, similar to that reported in Europe and North America; and confirmed a strong association with mixed cellularity subtype. Children <10 years of age showed a particularly high prevalence of EBV positive Hodgkin's disease (81%), with the following racial distribution 10/13 (76.9%) Black, 2/2 White, 1/1 Asian. 11/23 (47.8%) Black and 8/22 (36.3%) White adult cases were positive for EBV.

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DECLARATION

I declare that this dissertation other than the histological subtyping of the Hodgkin's Disease, is my own unaided work. It is being submitted for the degree of Master of Medicine (in the branch of Haematology) to the University of the Witwatersrand. It has not been submitted for any degree or examination in any other University.

Linda Kathleen Erasmus

August 1997

This research was approved by the Ethics Committee for Research on Human Subjects.

University of the Witwatersrand (Clearance Certificate No. 9/11/88).

**A COMPARATIVE STUDY OF HODGKIN'S DISEASE AT
BARAGWANATH AND JOHANNESBURG GENERAL HOSPITALS**

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A dissertation submitted to the Faculty of Medicine, University of the Witwatersrand, Johannesburg in partial fulfilment of the requirements for the degree of Master of Medicine in the branch of Haematology.

JOHANNESBURG, 1997

The fact that *in vitro* EBV induced growth transformation of any B cell is associated with Type III Latency gene expression, suggests that the co-ordinate action of multiple virus latent proteins are required for growth transformation.

Exactly what determines the pattern of gene expression in a given cell is uncertain, although cell transcriptional factors probably play a role. Different growth conditions may also influence differential activity of different promoters (Sinclair *et al*, 1994a).

Some cells seem to switch between latency types. Burkitt lymphoma cells show a characteristic Latency I gene expression on initial culture *in vitro*, but may switch to Type III Latency gene expression on serial passage (Rowe *et al*, 1987).

Reverse transcriptase PCR has shown that in healthy persons, a subpopulation of EBV-infected peripheral blood B lymphocytes, show Type I latency gene expression (Chen *et al*, 1995). As cellular and humoral responses to the full spectrum of latent proteins are stably maintained, some switching to Latency III must also occur (Murry *et al*, 1992a).

1.5.3 Viral Gene Expression in Latent Infection

EBNA 5 and EBNA 2 are the first viral proteins expressed in B lymphocyte infection. EBNA 2, in turn, transactivates cell genes (CD21 and CD23) and viral genes, including LMP-1 and LMP-2. CD23 in its full length is a B cell growth factor receptor, and in its truncated form is a B cell growth factor. Thus, up regulation of CD23 results in an autocrine growth loop. LMP-1 leads to expression of a number of cell adhesion and activation molecules including LFA 1, IFA 3, ICAM-1, CD21, CD40 and CD44 (see Figure 3). Cell DNA synthesis follows LMP-1 expression.

It is possible that the growth advantage conferred on Burkitt Lymphoma cells by virtue of deregulation of the *c-myc* oncogene, eliminates the need for expression of numerous EBV latent genes with growth transforming potential. Recent work has suggested that efficient expression of *c-myc* from certain Burkitt Lymphoma derived *c-myc*/Immunoglobulin gene fusions, may be EBNA 1 dependent (Magrath *et al.*, 1992).

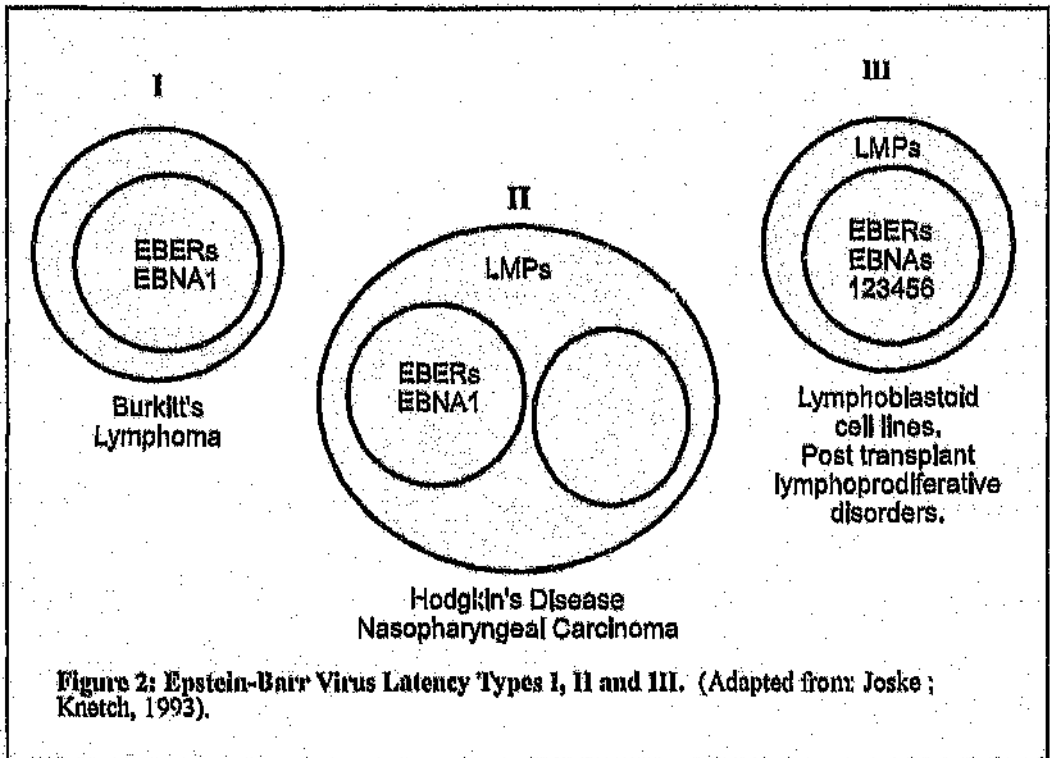
Latency II is found in HD and Nasopharyngeal carcinoma, and is characterised by expression of:

1. The EBER's.
2. EBNA 1 mRNA using the FQp promoter (as in Latency I).
3. LMP-1 and/or LMP-2A and/or LMP-2B mRNAs.
4. Bham H1A transcripts.

It is interesting that the levels of LMP-1 found in Reed-Sternberg cells are normally toxic to cells; suggesting that Reed-Sternberg cells are unusually tolerant.

Type III Latency is noted in lymphoblastoid cell lines as well as Post transplant B-cell lymphoproliferative disorders. It is characterised by expression of all the latency genes viz:

1. EBERs.
2. EBNAs 1-6.
3. LMP-1, LMP-2A and LMP-2B.
4. Bham H1A transcripts.



Latency I, is the most limited expression of EBV genes yet known. It was first described in EBV positive Burkitt lymphoma cells, but can also be reproduced by fusing lymphoblastoid cell lines with certain EBV negative haemopoietic cell lines.

The essential characteristics of Latency I are:

1. Expression of EBER's.
2. Transcription of EBNA 1, with activation of the FQp promoter, and silencing of the Cp Wp promoter which is active in Latency III.
3. Abrogation of LMP-1, 2A and 2B expression through silencing of the LMP promoters.

These patterns include expression of some or all of the following:

1. Two EBER's or EBV encoded RNA's. Small, nonpolyadenylated, noncoding transcripts. These are expressed in abundance. Approximately 10^6 per single viral copy (Clemens, 1993). Their function in EBV infection remains to be clarified.
2. 6 EBV Nuclear Antigens (EBNAs) - encoding mRNA's expressed from either the Wp, Cp or FQp promoter. The individual mRNA's encoding EBNA's 1-6, are generated from long primary transcripts by differential splicing. (Wolsetschlaeger *et al*, 1989). EBNA 2 is a transactivator of viral and cell gene products, and is essential for transformation. EBNA's 3 and 6 are also thought to be essential for virus mediated growth transformation.
3. mRNA's encoding the virus Latent Membrane Proteins; LMP-1, LMP-2A and LMP-2B. LMP-2B represents an N-terminal deleted form of LMP-2A. The same promoter region which controls expression of the leftward running predominant LMP-1 transcript, also controls that of the rightward running LMP-2B mRNA. The more abundant LMP-2A mRNA is expressed from another promoter (Laux *et al*, 1989). LMP-1 is essential for B lymphocyte growth transformation (Kayo *et al*, 1993).
4. A complex family of spliced, polyadenylated Bam H1A RNAs. It is uncertain whether these mRNAs are ever translated (Smith *et al*, 1993).

synthesis of RNA, expression of activation and adhesion molecules and secretion of immunoglobulin.

Initial data concerning the initial growth requirements for *in vitro* EBV infected cells (viz high serum concentration, feeder layers, cell density) suggests that these cells produce and are dependent on autostimulatory substances to promote cell growth. With continued cell proliferation these growth requirements diminish.

The initial growth requirements of EBV infected cells are similar to those of B lymphocytes stimulated to proliferate by antigen or cytokines. However, in B lymphocytes stimulated to proliferate for a short time *in vitro* by polyclonal B cell activators, EBV transformed B lymphocytes proliferate indefinitely in cell culture (Kieff, 1996).

1.5.2 Latency Types I, II and III

EBV latent infection is divided into 3 types, determined by characteristic patterns of latent gene expression. Each type of latency is in turn found in specific conditions (see Figure 2). EBV does not encode an RNA polymerase and uses host RNA polymerase II for transcription of viral mRNAs.

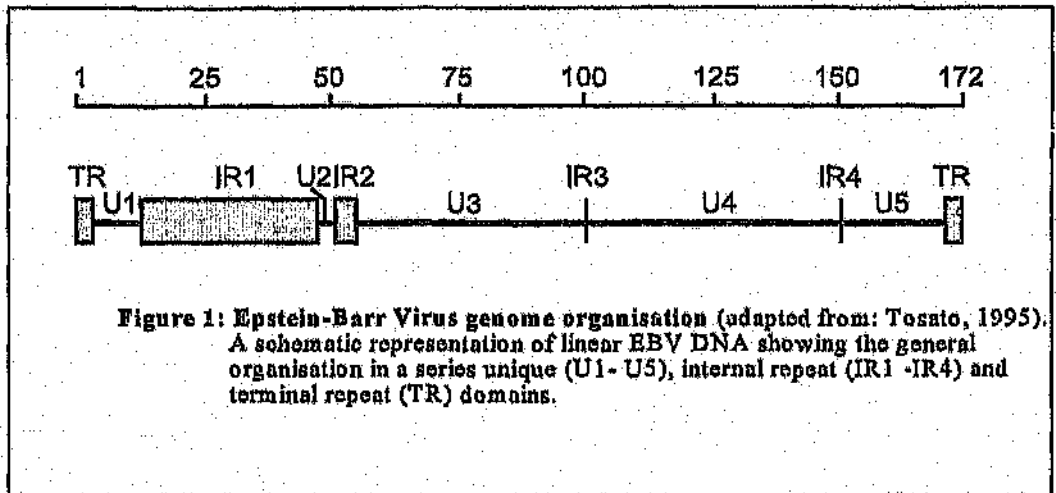
Evidence suggests that continued epithelial infection (manifest as lifelong viral shedding) may depend on seeding from the lymphoid reservoir (Gratama *et al*, 1988). The virus carrying B cell pool appears to be generalised throughout the body. *In situ* hybridisation of lymphoid tissue with EBER specific probes, shows that EBV carrying cells tend to be localised in extra follicular areas (Niedobitek *et al*, 1992).

In vitro, these latently infected B lymphocytes will either proliferate into long term lymphoblastoid cell lines or will undergo initial lytic infection. Virus released from the lytic infection will then transform other primary B lymphocytes in the culture (Kieff, 1996).

1.5.1 Latent Infection

In latent infection, EBV penetrates the cell, and remains present either as circular episomal DNA (formed through fusion of the terminal repeats) or, much less frequently, as linear DNA integrated into the host DNA. During mitosis, these episomes which are present in the host cell in low copy number, are copied by host cell DNA polymerase and passed to daughter cells. Latent EBV infection of B lymphocytes is associated with an ordered sequence of virus latent gene expression and results in continuous cell proliferation. The standard model of latent EBV infection is provided by *in vitro* transformed lymphoblastoid cell lines.

CD 21 is the receptor for the C3d component of complement. EBV binds to CD21 on the B lymphocyte cell surface via the major EBV outer envelope glycoprotein gp 350/220 (Frade *et al*, 1985). It is thought that an early event in EBV infection, possibly CD21 engagement, may activate the B lymphocyte before viral DNA transcription. This results in enlargement of the cell,



1.5 Viral Infection

Two forms of cellular infection are recognised : latent and lytic/replicative. In primary EBV infection, infection of the oropharyngeal epithelial cells, and epithelial cells lining the parotid duct, results in viral replication and release of infectious viral particles. Viral shedding is sustained for months after initial infection, then gradually diminishes, but is never completely eliminated (Morgan *et al*, 1979; Sixbey *et al*, 1983; Sixbey *et al*, 1984). Immunocompromised individuals show higher rates of viral shedding (Sumaya *et al*, 1986). It is unclear whether mucosal epithelium is the primary target for infection by orally transmitted virus or whether initial amplification in locally infiltrating B lymphocytes is required.

From its initial site of replication in the oropharynx, EBV then infects peripheral blood B lymphocytes. During acute infection, at least 1 in 10^4 circulating B lymphocytes is infected with EBV, these numbers diminish with time, but healthy EBV sero-positive individuals still carry approximately 1 in 10^6 EBV infected peripheral blood B lymphocytes (Rocchi *et al*, 1977). Infection of the B lymphocytes is predominantly latent, although some cells must at times be permissive for viral replication.

1.3 Viral Structure

Similarly to other herpes viruses, EBV consists of a protein core surrounded by DNA, an icosahedral capsid, and an outer envelope with external glycoprotein spikes. A protein tegument is found between the nucleocapsid and the envelope. The most abundant EBV envelope and tegument proteins are 350/220 and 152kd respectively (Kieff, 1996).

1.4 Genome Structure

The EBV genome is a linear, double stranded molecule of approximately 172 000 bp. A variable number of reiterated terminal repeats are situated at each end, and reiterated internal repeats divide the genome into short and long unique sequence domains. (See Figure 1).

When EBV establishes a latent infection in a proliferating cell, the terminal repeats fuse to form an extra-chromosomal episome. The number of terminal repeats is characteristic of that cell, and will be copied by host cell DNA polymerase and passed to daughter cells during mitosis. This provides a means of identifying monoclonal EBV in a cell population, as all progeny derived from a cell with a characteristic single viral fusion event, will have the same number of terminal repeats on restriction fragment length analysis. (Raab-Traub; Flynn, 1986).

The genome encodes an estimated 100 genes, but complicated differential splicing of RNA transcripts may result in production of many more proteins. (Kieff; Liebowitz, 1991).

(Henle *et al*, 1969). In contrast, in the USA and Western Europe, primary EBV infection may be delayed until adolescence or adulthood, sometimes resulting in the clinical syndrome of infectious mononucleosis (Henle *et al*, 1968 and 1970).

1.2 Classification of EBV

EBV is a member of the gamma herpes virus subfamily which includes both the *lymphocryptovirus* and *rhadinovirus* genera. Humans are the exclusive natural host for EBV (the prototype *lymphocryptovirus*), while closely related lymphocryptoviruses infect other old world primate species. Gamma herpes viruses establish latent infection in lymphocytes and are associated with cell proliferation (Kieff, 1996).

Two strains of EBV (Type 1 and 2) circulate in human populations. They differ structurally in the sequences of some of the latent viral genes (EBNA's 2,3,4,6, and to a lesser extent EBNA5) and biologically in their ability to immortalize B cells. (Rickinson *et al*, 1987; Sixbey *et al*, 1989). The genetic differences between EBV Type 1 and 2 are reflected in type common and type specific epitopes for antibody (Rowe, 1989).

EBV 1 appears to be more common in developed societies, and most EBV immune sera from these communities react preferentially or exclusively EBV 1 EBNA genes. Sera from African subjects show reactivity to EBV 1 and 2 epitopes in more equal proportions (Zimber *et al*, 1986; Sixbey *et al*, 1989). Although EBV 2 DNA can frequently be detected from oropharyngeal secretions in developed countries, recovery of EBV 2 from peripheral blood samples in these countries is unusual. This may reflect the less aggressive growth of EBV 2 - infected lymphocytes *in vitro* (Zimber *et al*, 1986; Young *et al*, 1987).

1.0 INTRODUCTION

1.1 History of Epstein-Barr Virus

Much of the interest in Epstein-Barr Virus (EBV) is fuelled by the virus's association with malignancy. Initially discovered in cultured lymphoblasts from samples of African Burkitt's lymphoma, EBV has subsequently been shown to be linked with a range of tumours, including Post transplant B-cell lymphoproliferative disorders, Nasopharyngeal Carcinoma (especially in Africa and China) and Hodgkin's disease (HD)

Despite serological and molecular evidence of an association between EBV and HD, the exact role of EBV in the aetiology of HD remains to be clarified. Patterns of viral latent gene expression differ between tumour types. In malignancies such as HD, where EBV expresses only a limited subset of latency genes, it is likely that EBV is just a contributing factor in the malignant process. On the other hand in Post transplant B-cell lymphoproliferative disorders, with a wider latency gene expression, EBV is likely to be the sole driving force behind malignant proliferation of cells.

Denis Burkitt, a missionary doctor working in East Africa in the 1950's, postulated the role of an infectious agent in the aetiology of Burkitt's lymphoma. Burkitt sent fresh tumour biopsies to Tony Epstein in London for screening of virus particles using electron microscopy. No virus could be demonstrated in freshly excised tumour biopsies, but Herpes virus like particles were subsequently identified in cultured cell lines (Rickinson and Kieff, 1996).

Subsequent seroepidemiological studies showed EBV to be widespread in human populations, with most adults having antibodies to the virus (Henle *et al*, 1969). In developing communities, primary EBV infection is usually an asymptomatic event, occurring within the first 3 years of life

LIST OF ABBREVIATIONS

ANOVA	-	Analysis of variance
DNA	-	Deoxyribonucleic Acid
EBV	-	Epstein-Barr Virus
EBER	-	EBV Encoded RNA
EBNA	-	EBV Nuclear Antigen
HD	-	Hodgkin's Disease
HHV-6	-	Human Herpes-Virus 6
HIV	-	Human Immunodeficiency Virus
HLA	-	Human Leucocyte Antigen
IHC	-	Immunohistochemistry
ISH	-	In-situ hybridisation
LMP	-	Latent Membrane Protein
PCR	-	Polymerase Chain Reaction
rt-PCR	-	Reverse Transcriptase Polymerase Chain Reaction
RNA	-	Ribonucleic Acid
Tdt	-	Terminal deoxynucleotidyl transferase
TRAF	-	Tumor Necrosis Factor Receptor Associated Factor

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1.7.2 Immunohistochemistry

This technique uses labelled monoclonal antibodies to stain individual cells for the presence of viral proteins. The ability to localise the viral product is obviously an advantage. In addition paraffin embedded tissue, even several years old can be used for immunohistochemistry (Pallesen *et al*, 1991). The commonest antibody is directed against LMP-1.

1.7.3 *In-situ* Hybridisation

In situ hybridisation also has the advantage of precise localisation of EBV within the cell. The technique uses labelled nucleic acid probes to bind to complementary viral DNA or RNA and can be performed on frozen or formalin fixed paraffin embedded sections (Khan *et al*, 1992). Although viral DNA was the initial target, the abundance of EBER transcripts ($\pm 10^3$ - 10^6 copies of EBER for every EBV genome) makes the signal to noise ratio more favourable despite the relative instability of RNA. The probes do not need to be radioactive for EBER. Another advantage of probing for EBER is that purely lytic infections are not detected (Gilligan *et al*, 1990).

Chromosome *in situ* hybridisation using floresceinated probes to viral DNA can be used to identify the extremely rare event of integration into host chromosomal DNA (Gulley *et al*, 1992). This is of little practical relevance.

1.7.4 Polymerase Chain Reaction

PCR amplification of viral DNA can be performed on paraffin sections and fine needle aspirations. PCR of viral DNA has two major disadvantages: viz that it is almost *too sensitive* and localisation of the EBV product is not possible. With a sensitivity of 1 or 2 target DNA sequences in

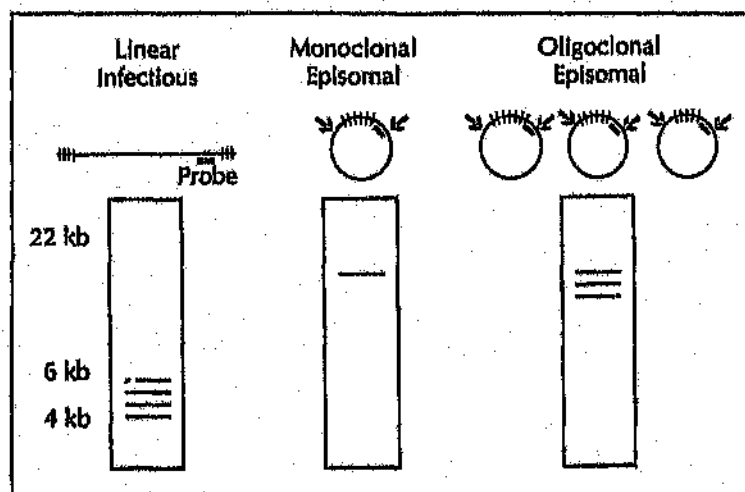


FIGURE 4: SOUTHERN BLOT ANALYSIS OF EPSTEIN-BARR VIRUS DNA STRUCTURE (adapted from Gulley, Raab-Traub, 1993).

This technique is based on varying numbers of terminal repeat sequences (hatch marks) at the end of linear viral DNA when digested with restriction enzymes, electrophoresed, transferred onto a nylon membrane and hybridized with a probe to be right terminus (bars). Linear infectious DNA is seen as a ladder array of small fragments (left). In contrast, Epstein-Bar virus-associated tumors contain viral episomes in which the terminal repeat sequences have fused to form a larger restriction fragment that is identical in all clonal progeny. Monoclonal tumors contain a single fused terminal fragment (center), while oligoclonal tumors contain multiple bands whose size depends on the number reiterations of the terminal repeat sequences (right). kb indicates kilobase.

1.7.1 Southern blot analysis

Southern blotting involves nucleic acid hybridisation of size fractionated DNA that has been immobilised on to nylon filter, and permits detection of viral DNA amongst the host DNA in a sample of human tissue.

The sensitivity of southern blotting depends on which region of the EBV genome is probed in the hybridisation step. At best, the sensitivity is approximately 1 EBV molecule for every 100 cells in the specimen. This allows for detection of EBV in EBV-associated tumors (eg post-transplant lymphoproliferative disorders) as well as in tissues containing abundant replicative virus. One of the advantages of southern blotting is that it can be used to determine the clonality of EBV infected cells. This is based on the principle that the number of terminal repeats present in the fused terminus of the EBV episome is unique to that cell, and will be passed onto its progeny.

Following digestion with a restriction enzyme, probes are used to detect the immobilised terminal restriction fragment of EBV DNA (Raab-Traub; Flynn, 1986). See Figure 4.

Infected tumours are represented by a single fused terminal restriction fragment, implying only that the virus was present before transformation and not necessarily that it drove the transformation process. Linear viral DNA characteristic of replicative infection is represented as a ladder-like arrangement of terminal restriction fragments.

Some cytotoxic T cell responses are EBV type specific, indicating that subtle changes in peptide sequence may abrogate recognition.

Occasionally sequence polymorphisms affecting cytotoxic T lymphocyte epitope regions, can occur between viral strains of the same type. A fascinating example is the HLA-A11 restricted epitope in EBNA 4 which is conserved among most Type 1 strains worldwide. However, in South East Asian populations and those of Papua New Guinea coastal regions, where the HLA A11 allele is unusually prevalent, all the Type 1 EBV strains are specifically mutated in key residues in this epitope. These mutant peptide sequences do not bind to HLA A11 molecules and cannot be recognised by HLA-A11 restricted cytotoxic T cells. It is tempting, therefore to speculate that virus with these mutations enjoyed a selective advantage in these populations. However, studies have detected similar mutations in virus isolates from the highlander population of Papua New Guinea in which the HLA A11 frequency is very low. It is thus possible that these mutations represent a founder effect rather than the result of selective pressure from the immune system (de-Campos-Lima *et al*, 1993; 1994).

1.7 Detection of EBV in Human Tissues using Molecular Genetic Techniques

Traditionally infectious EBV was detected by its ability to immortalise naive blood lymphocytes obtained from umbilical cord blood.

Recent developments in the application of DNA technology to detect EBV in human tissue, have contributed tremendously to new advances in the understanding of EBV related disease. These include immunohistochemistry as well as nucleic acid hybridisation techniques such as Southern blot analysis, *in situ* hybridisation to viral DNA or RNA and the polymerase chain reaction (PCR).

Current evidence suggests that CD8 positive T cells are important in limiting the expansion of latently infected B cells and may also target infected cells expressing lytic antigens, although much less is known about the latter.

Experiments from almost two decades ago (Moss *et al*, 1978) showed that cytotoxic T lymphocytes from EBV seropositive donor's circulating memory pool, reactivated *in vitro* were able to suppress the growth of lymphoblastoid cell lines. As expected, this cytotoxic T cell response was HLA class I restricted.

Any of the EBV antigens expressed in lymphoblastoid cell lines are potential sources of immunogenic peptide. It has been previously documented that the identity of peptide epitopes is significantly influenced by HLA type. Studies of a panel of virus immune donors showed that most individuals have strong cytotoxic T cell responses to more than one EBV latent antigen. In a high proportion of individuals, this response appears to be directed against epitopes from EBNA3, 4 and 6 latent proteins and in some donors also LMP-1 and LMP-2. To some extent, (but not entirely) this reflects epitope selection by HLA types common in Caucasian populations (Khanna *et al*, 1992; Murry *et al*, 1992a). In several of these studies, the specificity of large numbers of EBV specific cytotoxic T lymphocyte clones could not be identified, suggesting that other viral target antigens remain to be identified.

Interestingly enough, surveys of over 30 virus-immune donors failed to show any response against EBNA 1 epitopes. Whether this is an effect of study design, or EBNA 1 is truly protected from host immune response remains to be clarified.

responses. These include the BLLF 1 gene which encodes gp 350/220 - the dominant external virus glycoprotein that mediates virus binding to the B lymphocyte receptor. gp350/220 is also the most abundant viral protein in the lytically infected cell plasma membrane and most of the human EBV neutralising antibody response against this protein. The other late gene of interest is BCRF1 which shares nearly 90% co-linear identity in amino acid sequence with the human Interleukin 10 gene (Hsu *et al*, 1990).

BCRF1 is expressed only in late EBV replication, and although it has B cell growth factor activity, it appears to have no effect on growth transformation nor maintenance of latent infection (Swaminathan *et al*, 1993). However, these mutant BCRF1 recombinant infected cells differ from wild type recombinant infected cells in their inability to block gamma interferon release in cultures of permissively infected lymphoblastoid cell lines incubated with autologous human peripheral blood mononuclear cells. Thus, the role of BCRF1 in latent infection is probably to dampen natural killer and T cell cytotoxic response to EBV infection (Moore *et al*, 1990; de Waal-Malefyt *et al*, 1991).

1.6 Cell-mediated Immune Responses in EBV Carriers

Antibody responses to EBV in primary infection as well as in viral carriers are well described. It is unlikely that any of these antibodies play a role in the control for persistent infection once the carrier status has been established. However, anti-gp 350 antibodies are active against many EBV strains, and may contribute to protecting the host from a second EBV infection.

BZLF1 can down-regulate the EBNA Cp promotor-possibly facilitating the transition from latent to lytic infection. (Kenney *et al*, 1989). EBNA1 continues to be expressed as an early lytic infection gene product, under control of a down-stream promotor. BZLF1 also appears to associate with p53 and may inhibit p53 induced apoptosis in response to lytic EBV DNA replication. (Zhang *et al*, 1994).

1.5.6b Early Genes

This class includes several genes that are linked to DNA replication: including those encoding for DNA polymerase (BALF 5), major DNA binding protein, (BALF 2), thymidine kinase (BRLF 1) and others.

BHRF 1 gene encodes an abundant EBV early protein, HRF 1. HRF 1 shows extensive co-linear homology with bcl-2, (Cleary *et al*, 1986) and is thought to prevent apoptotic cell death in lytic EBV infection (Henderson *et al*, 1993). Deletions of BHRF1 ORF or stop codons early in BHRF1 does not affect the ability of the resultant mutants to initiate or maintain cell growth transformation (Marchini *et al*, 1991; Lee *et al*, 1992).

Similarly to some of the immediate early gene products, two EBV early gene products, two EBV proteins (BSML 1 and BMRF 1) may also transactivate expression of other early EBV genes.

1.5.6c Late Genes

Most of the known late EBV genes encode structural viral proteins required for successful packaging of viral DNA and formation of the virion. All the viral glycoprotein genes identified to date are also late genes. These are of interest because of their potential role in eliciting antibody

Two possible roles for EBERs have been proposed, based on structural similarities between Adenovirus VA and cell RNA V6 and the known functions of the latter two molecules viz. direct inhibition of an interferon induced protein kinase that would block translation or involvement in RNA splicing. No strong experimental evidence exists to support either of these theories (Swaminathan *et al*, 1992; Sharp *et al*, 1993).

1.5.6 Viral Gene Expression in Lytic Infection

In replicative infection, complete viral particles are released from cells which are lysed and die. In *in vitro* models of EBV infection all 3 forms of latency can be induced directly into lytic cycle. Chemical inducers such as phorbol are commonly used. In contrast to the latent phase of infection, when only a limited number of proteins are expressed, activation of the EBV lytic cycle is characterised by the presence of up to 80 virus specific RNA species.

Some virus genes are expressed early after induction, and are independent of new protein synthesis. These are classified as immediate early. Early lytic virus genes are expressed slightly later, and their expression is not affected by inhibition of viral DNA synthesis. Proteins are formally categorised as late, if inhibition of viral DNA synthesis results in a marked reduction in their expression. They are also sequentially expressed late.

1.5.6a Immediate Early Genes

These include BZLF1, BRLF1 and BFLF4 whose protein products are transactivators of early EBV lytic gene expression.

membrane, enables LMP-1's cytoplasmic domain to interact with growth factor receptor pathways resulting in constitutive activation. Experiments have shown that the LMP-1 cytoplasmic domain interacts with the tumour necrosis factor receptor signalling pathways (Mosialos *et al*, 1995).

1.5.4f LMP-2A and 2B

LMP-2A and 2B are sometimes referred to as terminal proteins 1 and 2 because their transcripts cross the terminal repeats. EBNA 2 is able to transactivate both LMP 2A and LMP 2B expression.

LMP-2 appears to co-localise with LMP-1 in the plasma membrane of latently infected B lymphocytes. LMP-2 expression in Burkitt Lymphoma lymphoblasts blocks the calcium mobilisation that normally follows cross-linking of surface immunoglobulin, CD19 or MHCII. This effect appears to be mediated through LMP-2A's interaction with B lymphocyte *src* family tyrosine kinases, particularly "*fyn*" and "*lyn*" (Miller *et al*, 1993). LMP-2A expression is also found to block the effect of surface immunoglobulin crosslinking on activation of lytic EBV infection presumably through a similar mechanism (Miller *et al*, 1994). This is probably critical in maintaining EBV persistence in humans.

1.5.5 EBER's

Although EBERs are by far the most abundant RNA species in latently infected cells, their role in infection remains to be clarified. EBER's are not translated and do not appear to be essential for transformation.

for EBNA 5 in directly/indirectly up regulating expression of autocrine growth factors essential for lymphoblastoid cell line outgrowth.

It has been shown that EBNA 5 together with EBNA 2 can induce G0-G1 transition. However, the exact mechanism involved, and its association of any with induction of a growth factor is unknown (Sinclair *et al*, 1994b).

1.5.4e LMP-1

LMP-1 is an integral membrane protein that is expressed in both latent and lytic infection.

LMP-1 can transform rodent fibroblasts, rendering them tumorigenic in nude mice (Wang *et al*, 1985). In addition, it can transform human keratinocytes and inhibit human epithelial cell differentiation (Fahraeus *et al*, 1990; Dawson *et al*, 1990). LMP-1 induces the expression of several lymphocytic activation markers and adhesion molecules, (Wang *et al*, 1988) and is also essential for primary B lymphocyte growth transformation (Kaye *et al*, 1993). LMP-1 has been shown to protect infected B lymphocytes from apoptosis through up regulation of bcl-2 (Henderson *et al*, 1991).

LMP-1 constitutively forms discrete patches in the plasma membrane, similar to those formed by growth factor receptors in response to ligand binding (Liebowitz *et al*, 1986).

The isolation of EBV recombinants specifically mutated in LMP-1 has allowed detailed analysis of the role of LMP-1 and its various domains, in B lymphocyte growth transformation. The current model for LMP-1's mechanism of action, suggests that LMP-1 patching in the plasma

EBNA 1 and LMP-1 are the only latent proteins that are also expressed during lytic infection.

1.5.4b EBNA 2

Many year ago, a transformation incompetent, laboratory mutant EBV was isolated, and shown to have a deletion of the DNA encoding EBNA 2 and the last two exons of EBNA 5 (Wang *et al*, 1987). Subsequent genetic analysis confirmed EBNA 2's essential role in B lymphocyte growth transformation (Cohen *et al*, 1989). As mentioned previously, EBNA 2 is specific transactivator of both cell and viral gene expression, and upregulates expression of LMP-1. Molecular genetic studies have shown a correlation between EBNA 2 sequences that are important for transformation and those that are important for transactivation of the LMP-1 promotor (Cohen *et al*, 1989).

Differences in EBNA 2 between EBV types 1 and 2, may account for their differing ability to transform B lymphocytes (Cohen *et al*, 1989; Rickinson *et al*, 1987).

1.5.4c EBNA's 3, 4 and 6

Molecular studies suggest that EBNA's 3 and 6 are essential for virus mediated lymphocyte growth transformation, but that EBNA 4 is not (Tomkinson *et al*, 1992; Tomkinson *et al*, *in press*). EBNA 6 upregulates CD21 mRNA and protein in non-EBV infected Burkitt Lymphoma cell lines *in vitro*.

1.5.4d EBNA 5

The exact role of EBNA 5 is not yet fully clarified. Its unusual nuclear localisation suggests that it may play a role in EBV RNA transcription or processing. Molecular genetic studies imply a role

Subsequent expression of EBNA 1, 3, 4 and 6 occurs. This may be related to the effects of EBNA 2 on EBNA 2 response elements. EBER expression lags behind that of the other latent proteins.

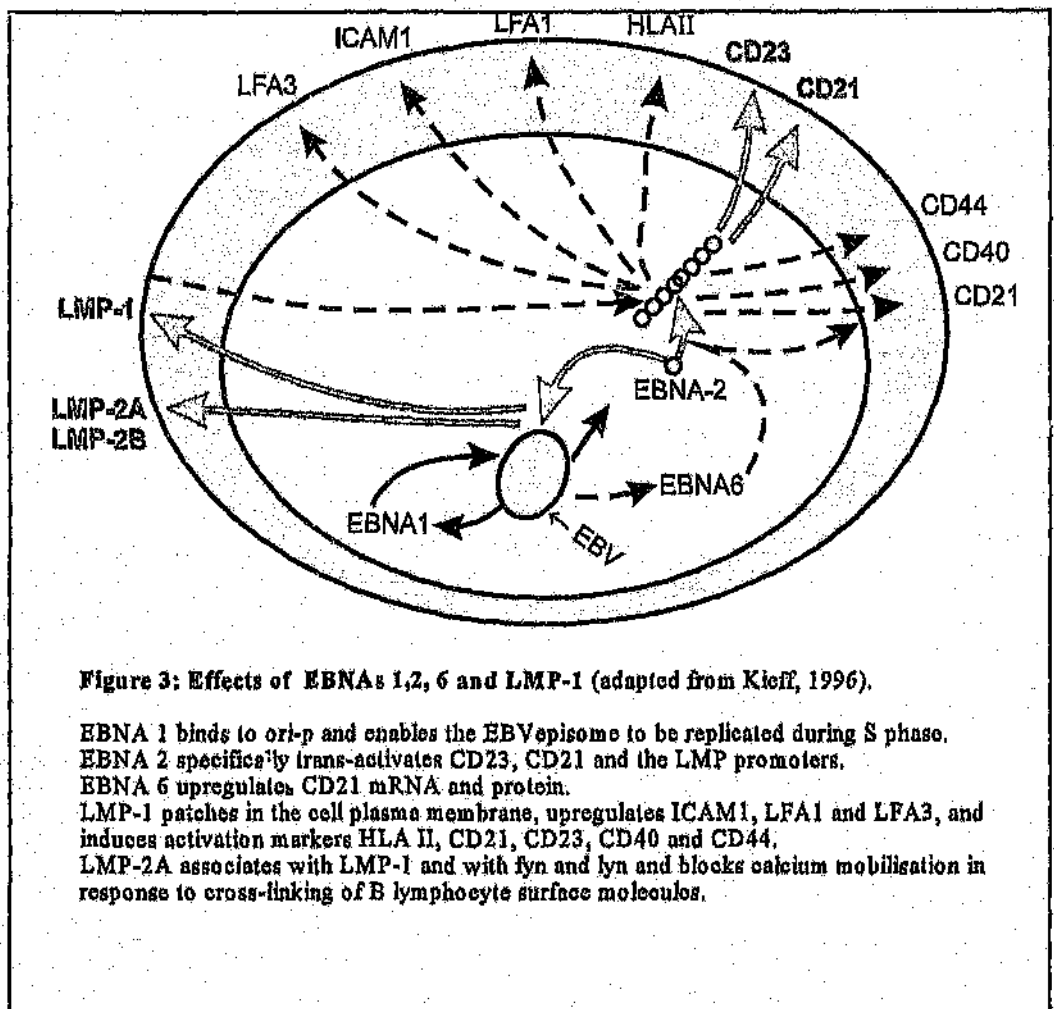


Figure 3: Effects of EBNA 1, 2, 6 and LMP-1 (adapted from Kieff, 1996).

EBNA 1 binds to ori-p and enables the EBV episome to be replicated during S phase.

EBNA 2 specifically trans-activates CD23, CD21 and the LMP promoters.

EBNA 6 upregulates CD21 mRNA and protein.

LMP-1 patches in the cell plasma membrane, upregulates ICAM1, LFA1 and LFA3, and induces activation markers HLA II, CD21, CD23, CD40 and CD44.

LMP-2A associates with LMP-1 and with fyn and lyn and blocks calcium mobilization in response to cross-linking of B lymphocyte surface molecules.

1.5.4 Latent Infection Proteins

1.5.4a EBNA 1

EBNA 1 binds to a viral origin of replication called ori-p and is necessary for episome persistence and replication in latently infected cells (Yates *et al*, 1984).

3.0 MATERIALS AND METHODS:

3.1 Epidemiological Data

Data regarding the number of patients admitted to the Johannesburg and Baragwanath Hospital Oncology units between January 1989 and December 1993, with a diagnosis of HL, was obtained retrospectively from relevant clinic files. Both paediatric and adult patients were included to give a total of 175 patients. Frequency tables were used to summarise the epidemiological data on these patients including age, race, sex and histological subtype, which was also obtained from these files.

3.2 Study Group: Archival Material

Formalin-fixed, paraffin embedded lymph node biopsies from the time of diagnosis were available on 61 of the above patients. These formed the study group.

For each of these cases, the histological subtype was jointly reviewed by 2 pathologists from the Anatomical Pathology Department, using immunohistochemistry when indicated. Both pathologists were blinded to the initial biopsy report.

These biopsies were then investigated for the presence of EBV products (LMP-1 and EBERs), as well as bcl-2 protein, using the techniques outlined below. Sections were only considered EBV or bcl-2 positive if Reed-Sternberg/Mononuclear variant cells were identified as containing these products. For both bcl-2 and LMP-1 expression, positivity was dependent on whether <10%, 10-50% or 50% of the Reed-Sternberg/Mononuclear variant cells present were positive.

2.0 AIMS AND OBJECTIVES OF STUDY

- 1(a) To investigate the prevalence of EBV in children and adults with HD at Baragwanath and Johannesburg General Hospitals from, January 1989 to December 1993, using:
 - a) Immunohistochemistry for detection of Latent Membrane Protein 1 (LMP-1) expression; and
 - b) In situ hybridisation for isolation of Epstein-Barr encoded RNAs (EBERs).
- 1(b) and to correlate LMP-1 positivity with Bcl-2 expression and apoptosis.
- 2) An epidemiological study of the above patients was conducted to establish the distribution of age, sex, race and histological subtype.

An early study using southern blot analysis failed to detect HHV-6 DNA sequences in cases of HD (Gledhill *et al*, 1991).

A more recent study, showed a high prevalence of HHV-6 infection in HD using PCR, but, in situ hybridisation showed that the HHV-6 positivity was restricted to lymphocytes while Reed-Sternberg cells and mononuclear variants were consistently negative (Valente *et al*, 1996).

It therefore seems unlikely that HHV-6 plays a specific role in the pathogenesis of HD.

(increased numbers of neoplastic cells). A 30-base-pair deletion in the carboxy-terminal domain of the LMP-1 gene was found in 89% of HIV infected patients vs 32% of ordinary HD. This deletion has been previously described and is thought to affect the rapid turnover of LMP-1, leading to accumulation of the protein within the cell (Sandvej *et al*, 1994).

Several investigations have shown that paediatric transplant patients undergoing primary EBV infection post transplant ran a higher risk of developing Post transplant B-cell lymphoproliferative Disorders than patients who were EBV sero-positive prior to transplant (Savoie *et al*, 1994). It will be interesting to see whether HIV positive patients in developing countries, who are likely to have been EBV sero-positive prior to HIV-related immunosuppression, show a different incidence of HD and/or different prevalence of EBV positivity when compared with HIV positive patients in developed countries.

1.9.8 Familial Hodgkin's Disease

An interesting recent study of cases of Familial Hodgkin's disease suggested that although serologically, Familial HD's patients had higher geometric mean antibody titres to viral capsid antigen and early antigen D, the lack of concordance of EBER1 expression and EBV serology among Familial HD cases within the same family, probably indicates that EBV does not play an important role in Familial HD (Lin *et al*, 1996).

1.9.9 HHV-6 : Another Viral Candidate in Hodgkin's Disease

Another candidate virus for involvement in HD is Human Herpes Virus 6 (HHV-6). Two studies reported elevated HHV-6 antibody levels in HD, one of which was a case control study (Biberfeld *et al*, 1988; Clark *et al*, 1990).

The prevalence of EBV positive HD in China and Malaysia was intermediate between that of Western and developing countries viz 60.7% and 61% respectively (Zhou *et al*, 1993; Peh *et al*, 1997). A further study of a Chinese population in Hong Kong showed a prevalence of 65% EBV positive HD (Chan *et al*, 1995).

Very few studies have addressed the influence of ethnic origin on EBV positivity. In Ambinder's series (1993), 3/4 children of Hispanic origin in the USA sample, showed EBV positivity. Multivariate analysis of a study of EBV positivity in 125 cases of HD from USA, Mexico City and Costa Rica, showed that the strongest predictor of EBV positivity was mixed cellularity histology, and that Hispanic ethnicity was strongly and independently associated with EBV positivity (Gulley *et al*, 1994). A study from Malaysia suggested that HD in Indians is more often EBV associated than other ethnic groups, but this was not statistically significant (Peh *et al*, 1997). This issue warrants further investigation. If ethnic differences do occur, they may be related to recognition of specific viral epitope: different HLA types.

1.9.7 The Association of EBV and Hodgkin's disease in Immunosuppressed Patients

Whether immunosuppression, particularly in Human Immunodeficiency Virus (HIV) positive patients results in any marked elevation in the incidence of HD remains to be seen. There is, however, evidence to suggest that those tumours which do arise in immunosuppressed patients are more frequently EBV positive (Uccin *et al*, 1990; Audoin *et al*, 1992).

In 1996, Bellas *et al* published a series of 24 HIV positive HD. These showed EBV positivity in 100% of cases. Compared with 56 cases of "ordinary" HD, the HIV-associated HD showed a higher prevalence of unfavourable histological subtype, and more aggressive morphology

1.9.5 Prognosis

At least two series suggest that EBV positivity in HD does not affect prognosis (Vestlev *et al*, 1992; Armstrong *et al*, 1992a; Oudejans *et al*, 1997). However, a recent study shows that EBV positivity may confer a more favourable prognosis in childhood HD (Sinclear-Smith *et al*, 1997).

1.9.6 Developed vs Developing Countries

Most of the early studies of EBV positive HD were done in Europe and North America. An interesting question is whether the prevalence of EBV positive HD would be different in less developed countries where asymptomatic primary EBV infection occurs at a much younger age. Initial data suggests that these areas have a higher prevalence of EBV positivity: A study from Peru of a primarily paediatric patient group, showed increased prevalence of EBV positivity in all age groups (94% overall) (Chang *et al*, 1993). Similar results were found in adult Mexican patients: 70% of HD were EBV positive, and all histological subtypes appeared to be strongly associated (Quintanilla-Martinez *et al*, 1995). Comparison of EBV positivity in nodal lesions of adult HD in French and Algerian patients demonstrated a higher rate of positivity in Algerian patients (72% vs 33%), with a stronger association with mixed cellularity than other histological subtypes (Belkaid *et al*, 1995). Paediatric HD in Honduras and the United States showed 100% and 36% EBV positivity respectively; both shared a strong association between EBV positivity and mixed cellularity subtype. However, in contrast to the USA, in Honduras other histological subtypes also showed a strong association with EBV positivity (Ambinder *et al*, 1993).

1.9.4 Age

Initial data reflecting the association of EBV positive HD and age, was conflicting. Some investigators failed to find any association between EBV positivity and age (Coates *et al*, 1993; Khan *et al*, 1993). In 1991, Gledhill *et al* found a statistically significant trend for an increase in EBV positivity with age. This prompted a larger study which also showed a significant association between age and HD with a peak incidence at 25-30 years and a second rise beyond the age of 45 years (Jarrett *et al*, 1991). These differences may reflect statistical errors due to small sample size, variations in methodology, or possibly geographical variation.

There are now a number of studies from various countries showing that EBV positive HD is common in the paediatric (<16 years) age group, particularly in children <10 years of age (Chan *et al*, 1995; Andriks *et al*, 1997; Peh *et al*, 1997). One series included 55 cases from the United Kingdom, Brazil and Saudi-Arabia, and showed no significant difference in EBV positivity by country (Armstrong *et al*, 1993). Others have suggested that paediatric cases of HD from developing countries have much higher rates of positivity than those quoted for Europe and North America (Ambinder *et al*, 1993; Chang *et al*, 1993; Weinreb *et al*, 1996a).

A recent study of childhood HD from 10 different countries, showed a range of EBV positivity from 50% in the United Kingdom, Egypt and Jordan to 88% from Iran, 90% from Greece and 100% from Kenya (Weinreb *et al*, 1996b).

TABLE 1: EBV IN HODGKIN'S DISEASE

Year	Number of Cases	Method of Detection	% EBV Positive	Reference
1987	21	Southern blotting	19% (all mono-oligoclonal)	Weiss <i>et al</i> , 1987
1989	16 5	Southern blotting ISH for EBV Nucleic Acids	19% (all monoclonal) 100%	Weiss <i>et al</i> , 1989
1989	28	Southern blotting	29%	Stroul <i>et al</i> , 1989
1991	48	PCR	67% (30 amplification cycles) 79% (after 60 amplification cycles)	Knotch <i>et al</i> , 1991
1991	84	IHC for LMP-1	48%	Pallensen <i>et al</i> , 1991
1991	47	IHC for LMP-1 PCR	38% 68%	Herbst <i>et al</i> , 1991
1991	95	Southern blot Hybridisation	45%	Jarrott <i>et al</i> , 1991
1991	23 - Typical HD 13 - LP subtype	PCR ISH for EBHR1 PCR ISH for EBHR1	52% 47.8% 15% 0%	Weiss <i>et al</i> , 1991
1994	48	IHC for LMP-1 ISH for EBHRs PCR	29% 44% 71%	Lauritzen <i>et al</i> , 1994
1994	6	Single - cell PCR	66.6%	Ren <i>et al</i> , 1994
1994	8	ISH for EBV DNA	87.5%	Valente <i>et al</i> , 1994
1994	172	IHC for LMP-1	27.9% 0%	Pinkus <i>et al</i> , 1994

1.9.3 Histological Subtype

One group found a statistically significant association between EBV positivity and mixed cellularity subtype, (Pallensen *et al*, 1991) and this subtype has tended to have high rates of positivity (up to 90%) in most series (Desol *et al*, 1992; Murray *et al*, 1992b). Approximately 30% of nodular sclerosis cases are EBV positive. As nodular sclerosis subtype occurs most commonly in young adults the age group where there is most epidemiological evidence for the aetiological infectious agent, this could militate against EBV being a candidate. The lymphocyte predominant histological subtype, now recognised as a separate B cell entity, generally has a negligible rate of EBV prevalence (Weiss *et al*, 1991).

et al, 1974). In addition, a retrospective study of patients with HD revealed that normal individuals with elevated antibody to EBV have an increased risk of developing HD (Mueller *et al*, 1989). Although these data raise the possibility that EBV is directly involved in the pathogenesis of HD, it remains possible that these studies reflect an underlying immune defect or are a marker for infection by another virus.

1.9.2 Molecular Studies

Molecular studies provided the key evidence to irrevocably link EBV with HD. In 1987, Weiss and colleagues reported the detection of EBV genomes in 4/21 cases of HD using Southern blot analysis. Subsequent studies using a variety of techniques, have shown that overall, 40-50% of HD in the Western World are EBV positive. (See Table 1). The majority appear to involve Type I virus.

The ability of *in situ* hybridisation and immunohistochemical techniques to enable specific localisation of the EBV products to the Reed-Sternberg and mononuclear variant cells, adds credibility to the association between EBV and HD. It is probable that the few background EBV positive reactive lymphocytes detected in some HD specimens, contributed to the high rates of EBV positivity noted in some PCR studies (Wu *et al*, 1990). In addition, several studies addressed the clonality of EBV genomes within HD by examining the viral terminal repeat sequences (Anagnostopoulos *et al*, 1989; Staal *et al*, 1989; Weiss *et al*, 1989). Although there were some conflicting results, in the majority of cases, the infected cells were found to be clonal with respect to EBV, suggesting that EBV infected a single cell which subsequently underwent clonal expansion.

1.9 EBV and Hodgkin's Disease

1.9.1 Epidemiological and Seroepidemiological Data

The above epidemiological findings formed the basis for 2 hypotheses regarding the aetiology of HD:

1. Multiple aetiology hypothesis, proposed by MacMahon (1966) suggests that HD represents a heterogeneous group of diseases (at least 3 entities) with distinct aetiologies, and further suggested that HD in young adults may be caused by an infectious agent.
2. Delayed exposure hypothesis suggests that the development of HD is related to late infection with a common childhood pathogen (Gutensohn; Cole, 1977).

Despite initial studies with negative or inconclusive results, recent data suggests that there is evidence for weak clustering of HD cases (Glaser, 1990). This could be consistent with an aetiology involving a virus with a long latent period, or alternatively, delayed exposure to a common but not ubiquitous virus (Jarrett, 1992).

In 1977, Paffenbarger *et al*, found that the risk of developing HD was lower in persons who had experienced more common contagious illnesses in childhood

The findings of a case control study ie. that cases with HD had twice the rate of infectious mononucleosis, not only lent support to an infectious aetiology but implicated EBV as a possible agent (Gutensohn; Cole, 1980).

Several studies have shown that patients with HD frequently have abnormally high levels of antibody to EBV-Viral-Capsid antigen and early antigens (Levine *et al*, 1971, Langenhuyzen *et*

temperate zones south of the equator (Ezowoda *et al*, 1995). In South Africa, data from the National Cancer registry (Sitas, 1992) suggests that HD has a lower frequency in the black population. However, this may be affected by underreporting as well as poor access to health care amongst this population group.

MacMahon (1966) drew attention to the bimodal nature of the age incidence curve for HD. The shape of this curve varies in different communities. In developing countries, a Type I pattern is seen: the first peak of the bimodal distribution occurs in childhood, with a second peak in older age groups. There is a low incidence in the third decade. The Type III pattern is seen in developed countries and is characterised by low rates in childhood, a pronounced peak in young adults and a second peak in older adults. An intermediate Type II pattern is found in Central Europe, rural areas of developed countries and in the Southern USA. This is thought to represent a transition between type I and III, probably as a result of improved socio-economic conditions (Correa and O'Connor, 1971).

The incidence rate of HD is higher in males than females, being most pronounced in childhood. This ratio decreases to below one in the young adult years, then increases with age to the late fifties. The ratio is substantially lower for the nodular sclerosis subtype than for other histological subtypes (Glaser and Jarret, 1996). Histological subtypes of HD also vary with age. Nodular sclerosis accounts for the young adult peak in incidence, while other subtypes grouped together show a gradually increasing incidence with increasing age. The majority of childhood cases in developing countries are of mixed cellularity subtype.

approximately 10^5 cells. PCR should pick up the occasional positive cell present in virtually all healthy EBV immune individuals. However, most series using PCR for the detection of EBV in lymphomas report fairly clear cut differences between reactive lymph nodes and EBV related tumors (Telenti *et al*, 1990).

Probably a bigger limitation is the inability to localise viral product, as EBV DNA in the occasional background lymphocyte may be amplified. Hopefully this will be overcome by the development of EBV specific in-situ PCR techniques.

PCR can also be used to distinguish different EBV strains (Jilg *et al*, 1990). A promising, relatively new technique for analysing gene expression is reverse transcriptase PCR (rt-PCR). Using the reverse transcriptase enzyme cDNA copies are made from viral RNA templates. These relatively stable DNA products are subsequently amplified by PCR. cDNA is distinguished from native DNA by designing primers that span splice sites (Brooks *et al*, 1992).

Each of these techniques has its advantages and disadvantages. While very sensitive, EBER *in situ* hybridisation is technically fairly difficult. It is likely that in the future rt-PCR may prove to be the quickest, easiest and cheapest method of detecting EBV.

1.8 Epidemiology of Hodgkin's Disease

HD is an uncommon malignancy. In the USA, it had an annual age-adjusted rate of 2.8/100 000 for 1987-91. Other than in Asia, where the incidence of HD appears to be much lower, HD incidence rates range from 1.5-4.5/100 000 for males, and from 0.9-3.0/100 000 for females (Glaser and Jarrett, 1996). HD appears to increase in frequency from the equatorial to the more

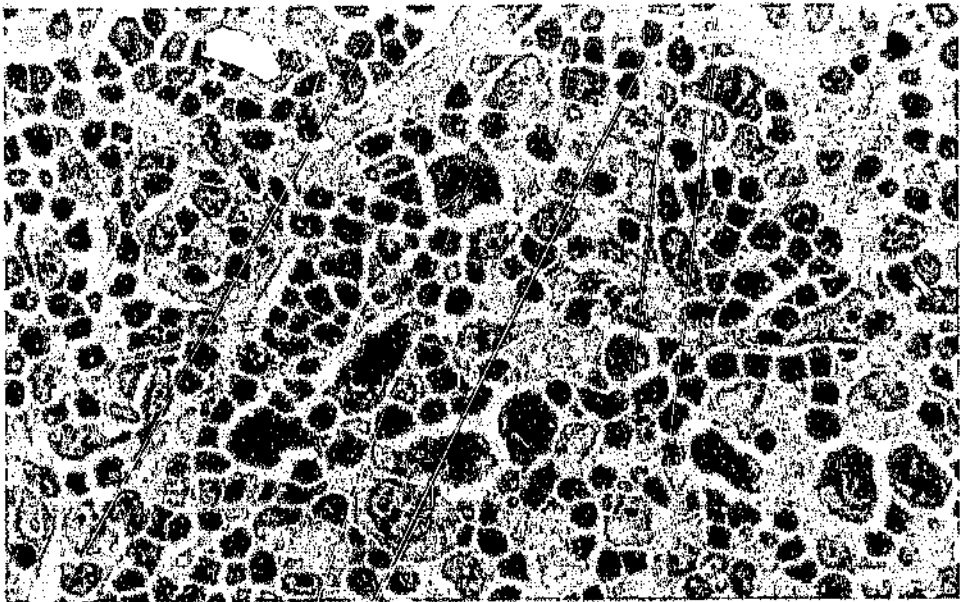


FIGURE 10: LYMPH NODE; HODGKIN'S DISEASE. MUMMIFIED CELLS ON H & E STAINING (X40)

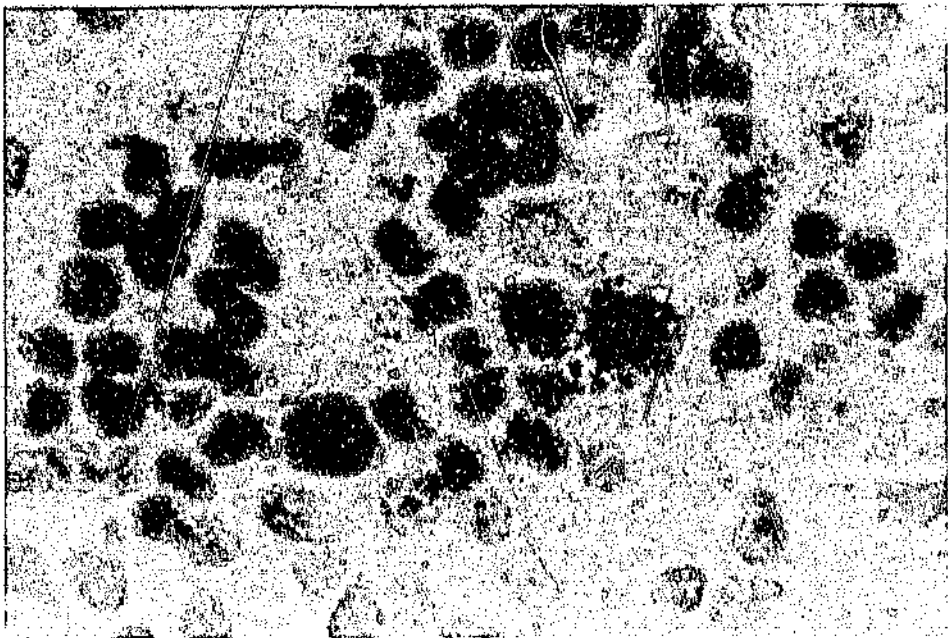


FIGURE 11: LYMPH NODE; HODGKIN'S DISEASE. APOPTAG (ONCOR, USA) SHOWING AN APOPTOTIC CELL ON THE LEFT AND A NECROTIC CELL ON THE RIGHT

After deparaffinisation, 4µm tissue sections cut onto sialyzed slides were digested with proteinase-K solution (200µg/ml in PBS; BOEHRINGER MANNHEIM, GERMANY) for 15 minutes at room temperature, then washed in water and quenched in 2% H₂O₂ in PBS buffer.

After application of equilibrium buffer for 1-30 minutes, Tdt enzyme (ONCOR, USA) was applied. The slides were covered with a plastic coverslip and left for 1 hour at 37°C. The slides were then incubated for a further 30 minutes in pre-warmed working strength stop/wash buffer, then washed in PBS.

Following application of anti-digoxigenin peroxidase for 30 minutes at room temperature, the slides were washed in PBS and stained with 0.1% DAB solution. After further washing in distilled water, slides were counterstained in methyl-green, dehydrated through 2x absolute ethanol and mounted in entellen mounting medium.

Diffuse brown staining of nuclear apoptotic bodies against a light green background was interpreted as a positive result.

Necrotic cells can in some instances contain stainable concentrations of DNA ends, but staining appears more diffuse than in their Apoptotic counterparts (see Figures 10 and 11).

Sections noted to have numerous apoptotic bodies on ApopTag were subsequently used as positive control. For the negative control, the Tdt enzyme was omitted. Controls were included in each batch.

3.6 ESTIMATION OF THE APOPTOTIC INDEX ON H& E

H&E stained tissue sections were scanned to identify areas with maximum numbers of mummified cells. Five such fields were then examined under 40x power, and the number of mummified cells and viable Reed-Sternberg/Mononuclear variant cells counted. The Apoptotic Index was calculated as the number of mummified Reed-Sternberg/Mononuclear variants divided by the total number of such cells multiplied by 100.

This process was repeated 3 times and an average figure calculated.

3.7 ESTIMATION OF THE APOPTOTIC INDEX ON APOPTAG

The areas identified on H & E as having many mummified cells, were marked on the corresponding ApopTag stained slide. The number of malignant cells identified by the stain as Apoptotic were then counted in 5 high power fields. This process was repeated 3 times and the Apoptotic Index calculated. Morphology of cells which were negative for ApopTag, were sometimes difficult to identify. Therefore, it was assumed that the denominator in the Apoptotic Index would be identical to that determined in the H&E sections.

3.8 *IN-SITU* APOPTOSIS DETECTION

In-situ apoptosis detection involves direct immunoperoxidase detection of digoxigenin-labelled genomic DNA. Residues of digoxigenin-labelled nucleotide are catalytically added to the numerous 3'-OH DNA ends generated during apoptosis by the enzyme Terminal deoxynucleotidyl transferase (Tdt). Standard immunoperoxidase detection techniques are implemented for visualisation.

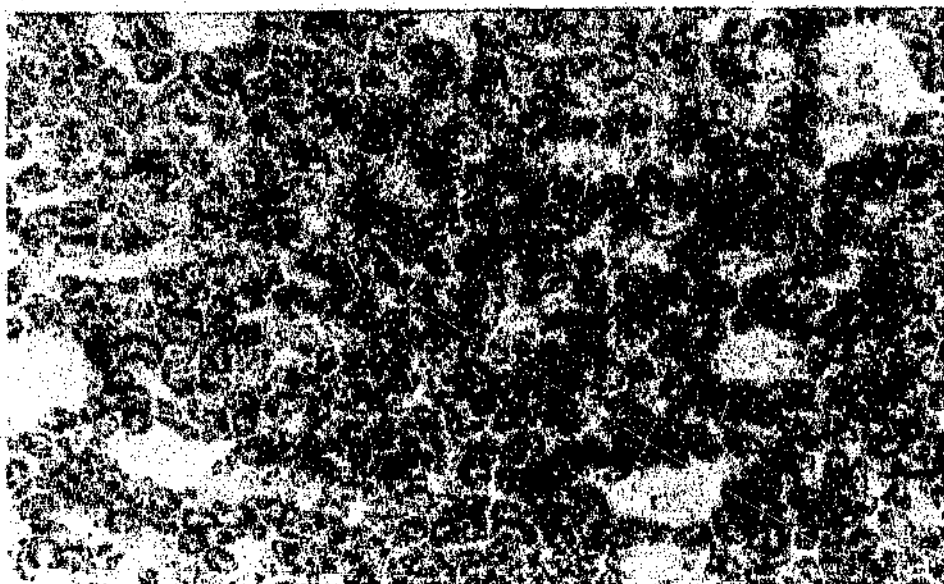
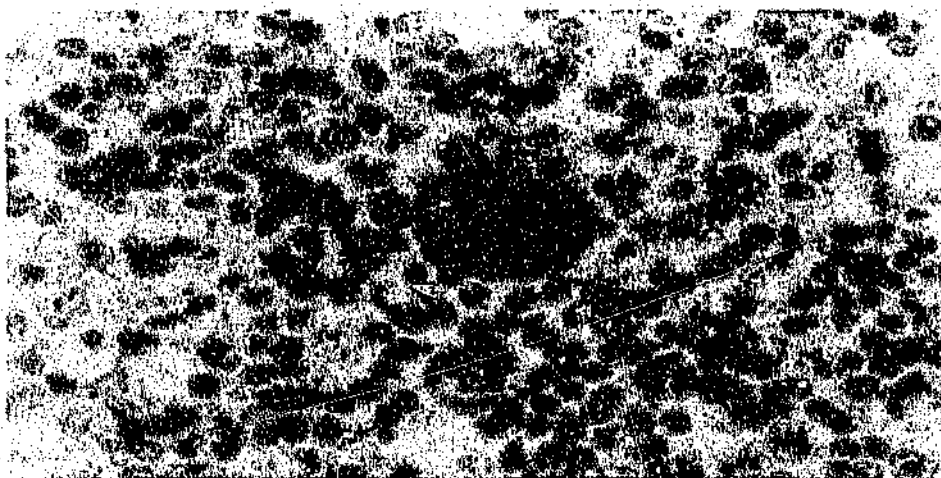


FIGURE 8: LYMPH NODE; FOLLICULAR B-CELL LYMPHOMA.

IMMUNOHISTOCHEMICAL STAINING FOR BCL-2. POSITIVE CONTROL
(X40).



**FIGURE 9: LYMPH NODE; HODGKIN'S DISEASE. IMMUNOHISTOCHEMICAL
STAINING FOR BCL-2 (X40) SHOWING POSITIVE STAINING IN THE
TUMOUR CELLS AND OCCASIONAL BACKGROUND LYMPHOCYTES**

TABLE 6: METHOD FOR MICROWAVE PRETREATMENT

0.01M Citrate Buffer pH=6.0.	
Solution A: 0.01M Citric acid	
	10.5g Citric acid in 500ml distilled water
Solution B: 0.1M Sodium citrate	
	2.94g Sodium citrate in 100ml distilled water
● 9ml of Solution A and 41ml of Solution B were mixed and made up to 500ml with distilled water (pH 6.0). ● Slides were placed in solution and boiled for 2 x 5 minutes in microwave at medium power. ● Slides were allowed to cool in solution for 20 minutes and then rinsed in PBS buffer at pH 7.6.	

3.5 IMMUNOHISTOCHEMISTRY FOR BCL-2

Immunohistochemistry was performed using monoclonal antibody directed against bcl-2 oncoprotein clone 124 (DAKO, DENMARK). As with LMP-1, 5% goat serum was used as a blocking agent and visualisation was achieved with a standard immunoperoxidase detection method.

4µm sections were dewaxed, rehydrated and microwave pretreated for antigen retrieval method (see Table 6).

After quenching with 3% H₂O₂ in methanol, and rinsing, slides were incubated with goat's serum, then incubated with anti Bcl-2 monoclonal antibody for 1 hour at room temperature. After rinsing with PBS, the slides were incubated with strep -Avidin-Biotin Complex/Horse radish Peroxidase kit (DAKO DENMARK). DAB was used as a substrate. The slides were counterstained with haematoxylin, dehydrated and mounted on entellin (MERCK, GERMANY).

Positive cells show strong brown granular cytoplasmic/membranous staining against a pale blue background (see Figure 9).

Positive and negative controls were included in each batch. A section of a Follicular lymphoma known to be bcl-2 positive, was used as a positive control (see Figure 8). For the negative control the primary antibody was omitted.

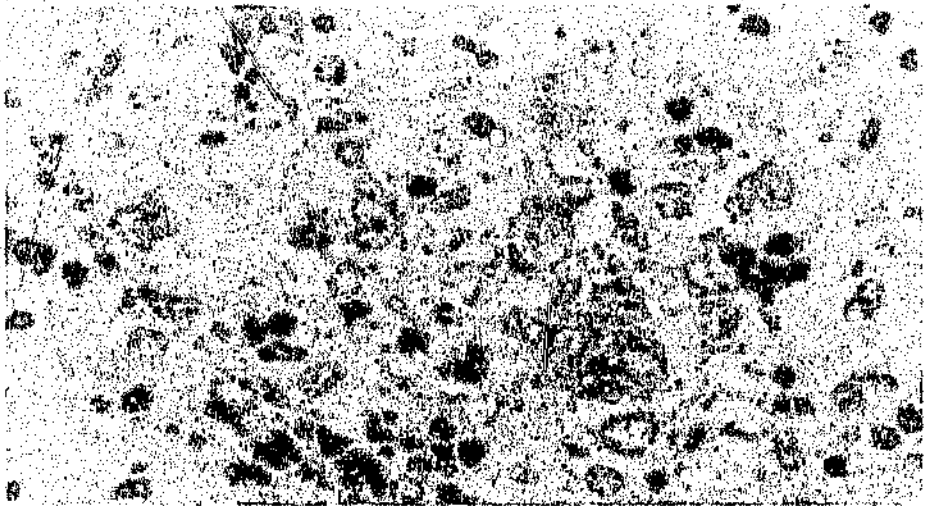


FIGURE 6: NASOPHARYNGEAL CARCINOMA. IMMUNOHISTOCHEMICAL STAINING FOR LMP-1. POSITIVE CONTROL (X40).

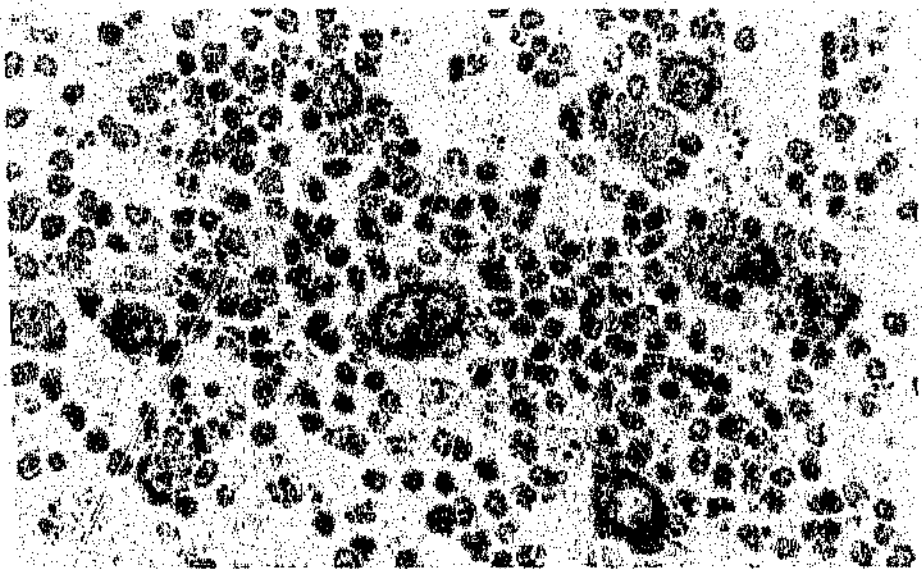


FIGURE 7: LYMPH NODE; HODGKIN'S DISEASE. IMMUNOHISTOCHEMICAL STAINING FOR LMP-1 (X40) SHOWING POSITIVE STAINING IN THE TUMOUR CELLS.

TABLE 4: COMPOSITION OF PHOSPHATE BUFFER SALINE (PBS)

PBS pH 7.6 was used over 48 hours.	
NaCl	16g
Na ₂ HPO ₄	3.5g in 2 litres distilled water
KCl	0.4g
KH ₂ PO ₄	0.4g
To ↑ pH add Na ₂ HPO ₄	
To ↓ pH add NaH ₂ PO ₄	

TABLE 5: METHOD FOR PREPARATION OF DAB SUBSTRATE

●	Prepared 10 minutes before use.
●	Mask and gloves were always utilised as DAB is carcinogenic.
●	0.1g DAB in 100mls PBS buffer was mixed on stirrer.
●	3 Drops of H ₂ O ₂ was mixed in 100mls distilled water. DAB solution was added before staining slides.
●	Slides were incubated in DAB/H ₂ O solution for 5 minutes then rinsed in running tap water for 10 minutes.
●	After staining the remaining DAB solution was neutralised in 5% bleach solution, left overnight and then discarded.

After rinsing in Phosphate Buffer Saline (PBS) buffer (see Table 4), slides were rinsed in 5% normal goat serum for 20 minutes, drained and then incubated with anti-LMP-1 monoclonal antibody (1:50; DAKO, DENMARK) for 1 hour and washed in PBS buffer.

The sections were then incubated with a biotinylated goat anti-mouse secondary antibody for 30 minutes. After rinsing in PBS buffer, Strept Avadin Biotin Complex/Horse Radish Peroxidase Duet kit (DAKO, DENMARK) was applied to the slides for a further 30-60 minutes. After washing, the slides were placed in Diamino-Benzidine-Tetrahydrochloride (DAB) solution (0.1% SIGMA, USA) (see Table 5), washed counterstained with haematoxylin, dehydrated and mounted with entellen (MERCK, GERMANY).

Interpretation of positive cells was achieved with brown cytoplasmic and/or membrane staining. A section of nasopharyngeal carcinoma known to be positive for both LMP-1 and EBER was used as a positive control (see Figures 6 & 7). For the negative control primary antibody was omitted and substituted with normal goat serum. These controls were included in each batch.

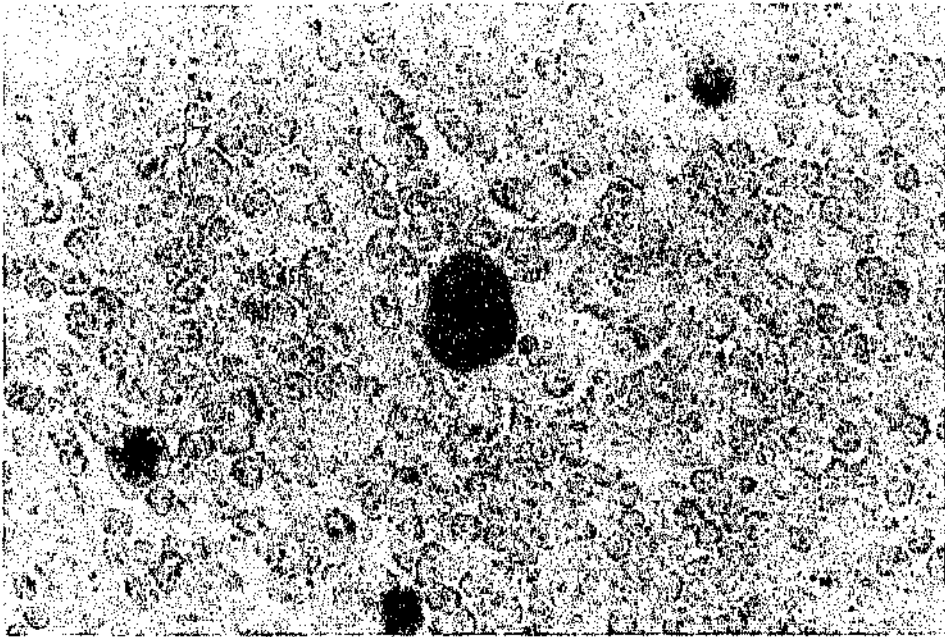


FIGURE 5: LYMPH NODE; HODGKIN'S DISEASE. *IN SITU* HYBRIDISATION FOR EBERS (X40) SHOWING A POSITIVE REACTION IN A MONONUCLEAR VARIANT CELL AND OCCASIONAL BACKGROUND LYMPHOCYTE.

3.4 IMMUNOHISTOCHEMISTRY FOR LMP-1

Immunohistochemistry was performed using DAKO-EBV LMP-CSI-4 mouse monoclonal antibody directed against EBV latent membrane protein 1 (LMP-1). Visualisation was achieved using a standard immunoperoxide detection technique.

4µm sections were dewaxed and rehydrated, then "digested" in pre-warmed trypsin solution (0.1% in PBS, SIGMA, USA) for 15 minutes at 37°C. The reaction was halted by rinsing in cold water. The slides were then incubated in quenching solution (3% H₂O₂ in methanol) for 30 minutes when necessary. This process removed any endogenous peroxidase.

positive for both EBV (LMP1 DAKO, Denmark) and EBER was used as a positive control. For the negative control oligonucleotide probe was omitted. These controls were included in each batch. All glassware, plastic ware and distilled water used for washing had to be free of contamination from RNase. This was achieved by washing in Diethyl Pyrocarbonate (DEPC) treated water and baking overnight at 180°C (Table 3).

TABLE 2: COMPOSITION OF TRIS BUFFER SALINE (TBS)

TBS (0.05M Tris, 0.15 NaCl), pH 7.6.
6.057g Tris (hydroxymethyl) amino methane.
8.766g NaCl in 1l distilled water.

TABLE 3: METHOD FOR PREPARATION OF DEPC WATER

- 1ml of DEPC was mixed in 1 litre sterilized water.
- DEPC solution was left overnight in a fume cupboard.
- Autoclaved for 20 minutes to inactivate the DEPC.
- TBS buffer was made up using DEPC treated water and was autoclaved once made.

In addition, an "Apoptotic Index" was estimated using morphological assessment of H & E sections complemented with special techniques (APOPTAG; ONCOR, USA).

3.3 *IN-SITU* HYBRIDISATION FOR EBV RNA (EBERs)

Each of the *in-situ* hybridisation and immunohistochemical techniques, utilized 4µm sections of the paraffin embedded, formalin-fixed tissue block.

The EBV RNA *in-situ* hybridisation studies were performed using oligonucleotides complementary to portions of the two nuclear EBER RNA's which are present in high copy numbers (~10⁶) in EBV infected cells. The oligonucleotides are a mixture of 5 30-mers, labelled with Fluorescein-isothiocyanate (FITC). 4µm sections were de-paraffinized, rehydrated and digested with proteinase K (500µg/ml; Boehringer Mannheim, Germany), in a humid incubator at room temperature for 20 minutes. After washing with pure water and 95% ethanol, the sections were then hybridised with the commercially prepared oligonucleotide probe (DAKO, Denmark) and incubated for 2 hours at 37°C. After washing the sections with Tris buffer saline (TBS), (see Table 2) signal was visualised using anti-Fluorescein-isothiocyanate-alkaline phosphatase (anti FITC/AP) detection kit using preprepared 5-bromo-4-chloro-3-indolyl phosphatase/Nitro blue tetrazolium (BCIP/NBT) Levamisole as a substrate (DAKO, Denmark, RNA detection kit). The sections were then washed and lightly counterstained with haematoxylin and mounted with entellen (MERCK, Germany).

A strong blue, black or blue/black colour in the nucleus/cytoplasm over background levels was considered a positive reaction. (see Figure 5) Clumping and margination against the nuclear membrane and nucleolus is sometimes noted. A section of nasopharyngeal carcinoma known to be

TABLE 15 (Continued)

BLACK PAEDIATRIC GROUP (≤ 15 Years)

Age/Race	Sex	LMP1	Subtype	Stage	Rx	Default	Outcome	Refractory/Relapse
Black paediatric	Male	Positive	MC	3	Chemo complete	-	Lost to follow up	Unknown
Black paediatric	Male	Positive	MC	2	Chemo complete	-	Alive	-
Black paediatric	Male	Positive	LD	4	Died before treatment	Not applicable	Died before Rx	N/A
Black paediatric	Female	Positive	MC	2	Chemo complete	-	Alive	-
Black paediatric	Female	Negative	NS	4	Chemo complete	-	Alive	-

SUBTYPES:

MC - Mixed Cellularity
 NS - Nodular Sclerosis
 LD - Lymphocyte Depleted

TREATMENT:

DXT - Radiotherapy
 ABMT - Autologous Bone Marrow Transplant

TABLE 15: DETAILS OF TREATMENT AND OUTCOME IN THE STUDY GROUP

BLACK PAEDIATRIC GROUP (≤ 15 Years)

Age/Race	Sex	LMP1	Subtype	Stage	Rx	Default	Outcome	Refractory/Relapse
Black paediatric	Female	Positive	MC	2	Chemo complete	-	Alive	-
Black paediatric	Female	Negative	NS	4	Incomplete chemo & DXT	-	Dead - unrelated	-
Black paediatric	Male	Positive	NS	2	Chemo complete	-	Alive	-
Black paediatric	Male	Positive	MC	2	Chemo complete	-	Alive	-
Black paediatric	Male	Positive	NS	4	Chemo complete	-	Dead - unrelated	Refractory
Black paediatric	Male	Positive	NS	3/4	Chemo complete	-	Alive	-
Black paediatric	Male	Positive	NS	2	Chemo, DXT, ABMT	-	Alive	Relapsed/Refractory
Black paediatric	Male	Positive	MC	3	Chemo complete	-	Alive	-

SUBTYPES:

MC - Mixed Cellularity
 NS - Nodular Sclerosis
 LD - Lymphocyte Depleted

TREATMENT:

DXT - Radiotherapy
 ABMT - Autologous Bone Marrow Transplant

Statistical analysis of outcome in LMP-1 positive and LMP-1 negative cases was not possible primarily due to small sample size exacerbated once stage and histological subtype was taken into account. The high loss to followup in certain centres and irregular clinic attendance/treatment further complicated the issue. Further, treatment policies differed somewhat between centres.

Data was available on 52 of the 61 patients in the study group and is summarised in Table 15.

In the paediatric group: 3 patients were EBV negative: 1 died of sepsis, (stage IV) 1 relapsed with refractory disease (stage II) and required autologous bone marrow transplant and has been alive and disease free for 6 months, and 1 (stage IV) has been alive for 7 years. Of the EBV positive cases 10 are alive and disease free: 7 stage II, 3 stage III (average time ~ 4 years 2 months) 1 was lost to follow up, 1 died before treatment started, and another had refractory disease and died of sepsis.

Of the adult cases, information was available on 36 of the 45 patients in the study group. 9 defaulted and were lost to follow up before their chemotherapy was completed.

Of the remaining 18 EBV negative patients: 8 (5 stage II, 3 stage III) are alive and disease free; 3 (1 stage II, 2 stage III) died of unknown causes, 6 had refractory disease/relapse (1 stage I, 3 stage II; 1 stage III; 1 stage IV) 3 are still alive, 2 lost to follow up and 1 died cause unknown. 1 refused hospital treatment and died presumably due to disease. Of the EBV positive cases, 3 completed chemotherapy and were still alive at least 6 months past therapy (2 were subsequently lost to follow up) 2 stage II and 1 stage III. 4 relapsed/had refractory disease (1 stage I, 2 stage II, 1 unknown); 2 of these were lost to follow up one is alive and 1 died (cause unknown).

Statistical analysis of outcome in LMP-1 positive and LMP-1 negative cases was not possible primarily due to small sample size exacerbated once stage and histological subtype was taken into account. The high rates to followup in certain centres and irregular clinic attendance/treatment further complicated the issue. Further, treatment policies differed somewhat between centres.

Data was available on 52 of the 61 patients in the study group and is summarised in Table 15.

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Of the adult cases, information was available on 36 of the 45 patients in the study group. 9 defaulted and were lost to follow up before their chemotherapy was completed.

Of the remaining 18 EBV negative patients: 8 (5 stage II, 3 stage III) are alive and disease free; 3 (1 stage II, 2 stage III) died of unknown cause, 6 had refractory disease/relapse (1 stage I, 3 stage II; 1 stage III; 1 stage IV) 3 are still alive, 2 lost to follow up and 1 died cause unknown. 1 refused hospital treatment and died presumably due to disease. Of the EBV positive cases, 3 completed chemotherapy and were still alive at least 6 months past therapy (2 were subsequently lost to follow up) 2 stage II and 1 stage III. 4 relapsed/had refractory disease (1 stage I, 2 stage II, 1 unknown); 2 of these were lost to follow up, one is alive and 1 died (cause unknown).

**TABLE 13: 2X2 SUMMARY TABLE SHOWING THE DISTRIBUTION OF BCL-2
POSITIVITY ACCORDING TO HISTOLOGICAL SUBTYPE**

Bcl-2	MC	NS	LD	Totals
Bcl-2 Positive	13	20	1	34
Bcl-2 Negative	14	12	1	27
Totals	27	32	2	61

MC = Mixed Cellularity
LD = Lymphocyte Depleted

NS = Nodular Sclerosis

**TABLE 14: 2 X 2 SUMMARY TABLE SUMMARISING THE DISTRIBUTION OF
LMP-1 AND BCL-2 POSITIVITY**

LMP-1	Bcl-2 Positive	Bcl-2 Negative	Totals
LMP-1 Positive	15	17	32
LMP-1 Negative	19	10	29
Totals	34	27	61

**TABLE 11: 2X2 SUMMARY TABLE SHOWING THE DISTRIBUTION OF LMP-1
POSITIVITY ACCORDING TO HISTOLOGICAL SUBTYPE**

LMP-1	MC	NS	LD	Totals
LMP-1 Positive	21	10	1	32
LMP-1 Negative	6	22	1	29
Totals	27	32	2	61

MC = Mixed Cellularity
LD = Lymphocyte Depleted

NS = Nodular Sclerosis

**TABLE 12: 2X2 SUMMARY TABLE SHOWING THE DISTRIBUTION OF BCL-2
POSITIVITY IN AGE/RACE SUBGROUPS**

Bcl-2	BA	BP	WA	WP	AP	Totals
Bcl-2 Positive	15	7	11	1	0	34
Bcl-2 Negative	8	6	11	1	1	27
Totals	23	13	22	2	1	61

BA = Black Adult
WA = White Adult
AP = Asian Paediatric

BP = Black Paediatric
WP = White Paediatric

TABLE 9: 2X2 SUMMARY TABLE SUMMARIZING THE DISTRIBUTION OF HISTOLOGICAL SUBTYPE ACCORDING TO AGE/RACE IN THE STUDY GROUP (n=61)

Age/Race	Subtype	Subtype	Subtype	Totals
	MC	NS	LD	
Black Adult	8	15	0	23
Black Paediatric	6	6	1	13
White Adult	11	10	1	22
White Paediatric	2	0	0	2
Asian Paediatric	0	1	0	1
Totals	22	18	2	42

MC = Mixed Cellularity
LD = Lymphocyte Depleted

NS = Nodular Sclerosis

TABLE 10: 2X2 SUMMARY TABLE SHOWING THE DISTRIBUTION OF LMP-1 POSITIVITY IN EACH AGE/RACE SUBGROUP (n=61)

LMP-1	BA	BP	WA	WP	AP	Totals
LMP-1 Positive	11	10	8	2	1	32
LMP-1 Negative	12	3	14	0	0	29
Totals	23	13	22	2	1	61

BA = Black Adult
WA = White Adult
AP = Asian Paediatric

BP = Black Paediatric
WP = White Paediatric

TABLE 7: 2X2 SUMMARY TABLE SUMMARIZING THE DISTRIBUTION OF AGE/RACE IN THE STUDY GROUP (n=61)

Age/Race	Number	Percentage
Black Adult	23	37.70
Black Paediatric	13	21.31
White Adult	22	36.06
White Paediatric	2	3.27
Asian Paediatric	1	1.63

TABLE 8: 2X2 SUMMARY TABLE SUMMARIZING THE DISTRIBUTION OF HISTOLOGICAL SUBTYPE IN THE STUDY GROUP (n=61)

Subtype	Number	Percentage
Mixed cellularity	27	44.26
Nodular Sclerosing	32	52.45
Lymphocyte Depleted	2	3.27

Bcl-2 was noted to be positive in 34/61 (55.7%) of cases, distributed as follows: 9 (<10%) Reed-Sternberg/Mononuclear variant cells positive, 12 (10-50%) Reed-Sternberg/Mononuclear variant cells positive, 13 (>50%) Reed-Sternberg/Mononuclear variant cells positive.

The distribution of bcl-2 positivity according to age/race and subtype are depicted in Tables 12 & 13 respectively. 15/23 (65.2%) and 11/22 (50%) Black and White adult cases respectively, showed bcl-2 positivity. 7/13 (53.8%) Black, 1/2 (50%) White and 0/1 Asian paediatric cases were bcl-2 positive. Regarding histological subtype: 1/1 (100%) lymphocyte depleted, and 20/32 (62.5%) nodular sclerosing and 13/27 (48%) cases of mixed cellularity subtype were bcl-2 positive.

Table 14 depicts the interaction between LMP-1 and bcl-2. Although there appears to be a negative association between LMP-1 and bcl-2, the chi-squared test shows that this is not statistically significant ($p \sim 0.15$).

Only 3 cases show both LMP-1 positivity and bcl-2 positivity in >50% of the malignant cells.

4.0 RESULTS:

4.1 STUDY GROUP:

The study group comprised 61 patients: 23 black adults, 22 white adults, 13 black paediatric, 2 white paediatric and 1 Asian paediatric patients. (See Table 7).

27 Cases were diagnosed as mixed cellularity subtype; 32 nodular sclerosing subtype; and 2 lymphocyte depleted subtype. (See Table 8).

The distribution of the various subtypes in relation to age/race is depicted in Table 9.

52% of the cases (32/61) were LMP-1 positive: of these 4 showed positivity in <10% of the Reed-Sternberg/Mononuclear variant cells, 5 showed positivity in 10-50% of Reed-Sternberg/Mononuclear variant cells and 23 showed positivity in >50% of Reed-Sternberg/Mononuclear variant cells. The prevalence of LMP-1 positivity according to subtype and age/race is depicted in Tables 10 & 11. Mixed cellularity histological subtype showed the highest prevalence of LMP-1 positivity 21/27 (77.7%), while 1/2 (50%) cases of lymphocyte depleted and 10/32 (31.2%) cases of nodular sclerosing subtype were LMP-1 positive.

The paediatric groups showed a high prevalence of LMP-1 positivity: 10/13 (76.9%) Black, 2/2 (100%) White and 1/1 (100%) Asian paediatric cases were LMP-1 positive. Regarding the adult cases: 11/23 (47.8%) Black and 8/22 (36.3%) White adult cases demonstrated LMP-1 positivity.

EBER positivity was noted in 24/61 cases, all of these were LMP-1 positive (ie 8 LMP-1 positive cases were EBER negative).

The agreement between the two methods for measuring the Apoptotic Index was assessed using the procedure outlined in Bland and Altman (1986) viz; a scatter plot of one method vs the other, and a plot of the difference vs the mean of two methods.

Epidemiological data regarding the total 175 patients was obtained retrospectively from patient files and summarised using frequency tables.

No statistical analysis of the study group regarding outcome was possible, due to the small numbers involved as well as the high rate of loss to follow up. This data is depicted in table form in the Results section.

3.9 STATISTICAL ANALYSIS

2x2 summary tables were used to summarise the distribution of the study group (n=61) with respect to age/race and subtype. Similar tables were used to summarise the prevalence of LMP-1, EBER and bcl-2 positivity in this group. LMP-1 and bcl-2 positive cases were also analysed according to the percentage of malignant cells noted to be positive.

Frequency tables were used to summarise the prevalence of LMP-1 and bcl-2 positivity within the age/race and subtype subgroups, and to summarise concordance between LMP-1 and EBER positivity.

The interaction between LMP-1 and bcl-2 was tested using the chi-squared test.

Histograms were used to summarise the distribution of the Apoptotic Index established on 61 cases by observation of mummified cells on the H&E stain, and on 42 cases using the ApopTag kit. The mean and median Apoptotic Index and standard deviation were noted.

The means of the Apoptotic Indices within each subtype and each age/race subgroup were compared using the Analysis of variance (ANOVA) and depicted graphically in box and whisker plots.

A two-way ANOVA was used to compare the means of the Apoptotic Indices by subtype and LMP-1 positivity as well as by subtype and bcl-2 positivity.

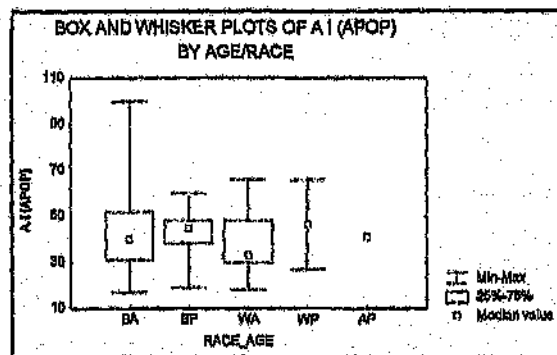
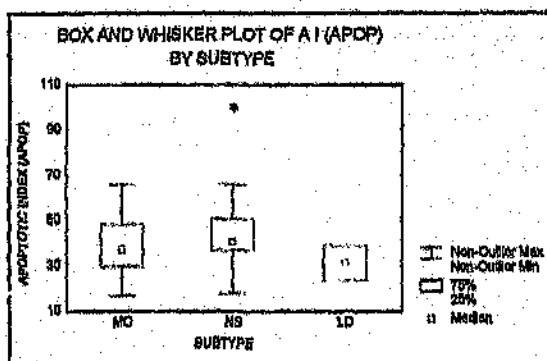


FIGURE 17: BOX AND WHISKER PLOTS OF APOPTOTIC INDEX ON APOPTAG BY AGE/RACE

BA = Black Adult
WA = White Adult
AP = Asian Paediatric

BP = Black Paediatric
WP = White Paediatric

The Between group ANOVA of Apoptotic Index (on ApopTag) by Age/Race gave a p-value of 0.56.

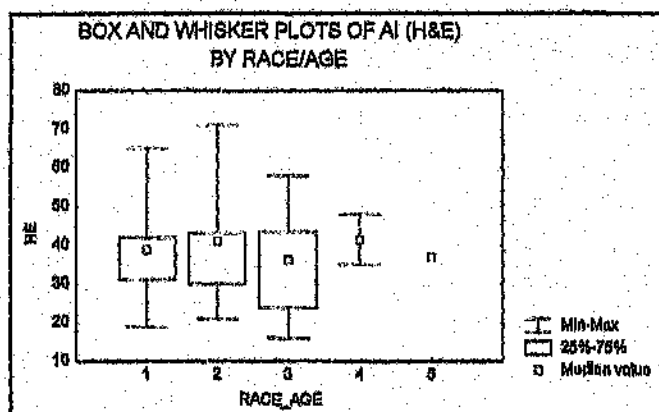


**FIGURE 16: BOX AND WHISKER PLOTS OF APOPTOTIC INDEX ON APOPTAG
BY SUBTYPE**

MC = Mixed Cellularity
LD = Lymphocyte Depleted

NS = Nodular Sclerosis

The Between group ANOVA of Apoptotic Index (on ApopTag) by Subtype gave a p-value of 0.33.



**FIGURE 15: BOX AND WHISKER PLOTS OF APOPTOTIC INDEX ON H & E
BY RACE/AGE**

- | | | | | | |
|---|---|------------------|---|---|------------------|
| 1 | = | Black Adult | 4 | = | White Paediatric |
| 2 | = | Black Paediatric | 5 | = | Asian Paediatric |
| 3 | = | White Adult | | | |

The Between group ANOVA of Apoptotic Index (on H & E) by Age/Race gave a p-value of 0.66.

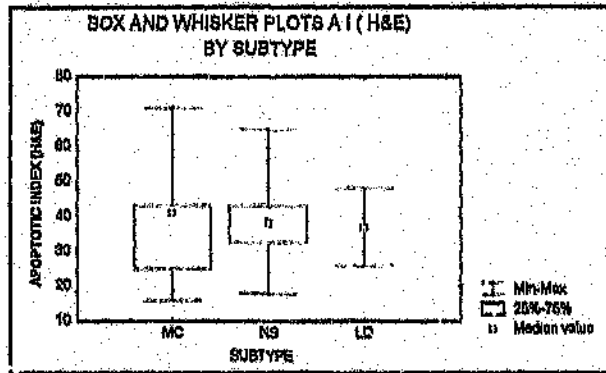


FIGURE 14: BOX AND WHISKER PLOTS OF APOPTOTIC INDICES ON H & E BY SUBTYPE

MC = Mixed Cellularity
LD = Lymphocyte Depleted

NS = Nodular Sclerosis

The Between group ANOVA of Apoptotic Index (on H & E) by Subtype, gave a p-value of 0.84.

Although the box and whisker plot of Apoptotic Index (established on H&E) by subtype and LMP-1 (see Figure 18) appeared to show an interaction, the ANOVA showed that this was not statistically significant ($p = 0.13$). This may be due to the small number of cases.

In particular, there was no statistically significant difference in the Apoptotic Index (on H&E) between LMP-1 positive and LMP-1 negative cases ($p = 0.58$). Although it appears that LMP-1 does not act by inhibiting apoptosis - again, this may be related to small sample size.

The apparent interaction between Apoptotic Index (on H&E) by bcl-2 and subtype on the box and whisker plot (see Figure 19) were similarly not statistically significant ($p = 0.22$) - possibly due to sample size. Contrary to expectation, Apoptotic Index on H & E did not differ significantly between bcl-2 positive and negative cases ($p = 0.19$), although again this may reflect sample size.

Analysis of Apoptotic Index (established using ApopTag) by LMP-1 and subtype also shows no statistically significant interaction ($p = 0.73$) (see Figure 20). These results also apply to bcl-2 and subtype ($p = 0.73$) (see Figure 21), and are possibly as a result of small sample size.

Similarly, analysis of Apototic Index (on ApopTag) by LMP-1 shows no statistically significant difference between LMP-1 positive and LMP-1 negative cases ($p=0.07$). Nor is any statistically significant difference in Apoptotic Index (on ApopTag) noted for bcl-2 positive and negative cases ($p=0.45$).

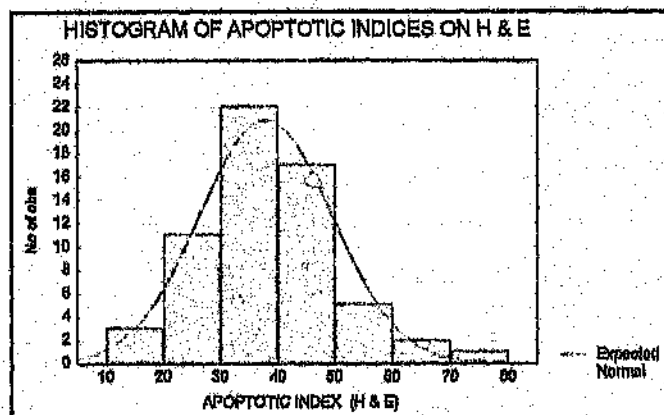


FIGURE 12: HISTOGRAM OF APOPTOTIC INDICES ON H & E.

Descriptive statistics for Apoptotic Index (H & E)

No obs	Mean	Median	Min	Max	SD
61	37.87	38.00	16	71	11.6

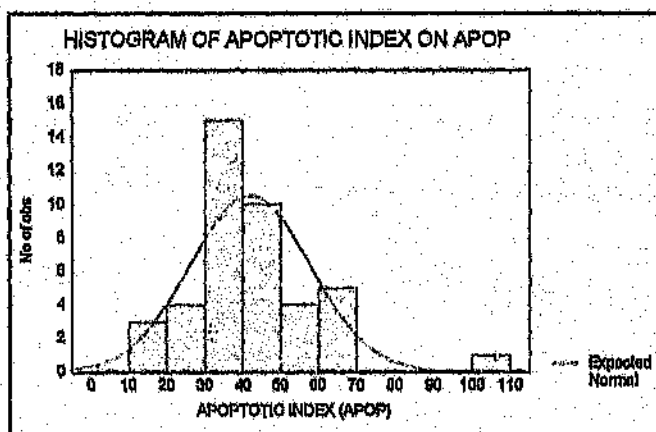


FIGURE 13: HISTOGRAM OF APOPTOTIC INDICES ON APOPTAG.

Descriptive statistics for Apoptotic Index (APOPTAG)

No obs	Mean	Median	Min	Max	SD
42	41.88	39.0	17.0	100.0	15.85

4.2 APOPTOTIC INDEX (AI)

On morphological assessment of H&E stained sections, ($n = 61$), the mean Apoptotic Index was 37.87% and the median Apoptotic Index 38% (SD = 11.6) (see Figure 12). The similarity between the mean and median suggests that the distribution of Apoptotic Index is symmetric. Figure 12 illustrates that the normal distribution provide a good fit to the data.

Figure 13 illustrates similar data for the Apoptotic Index determined on staining with ApopTag. Although the mean (41.88%) and median (39.0%) are similar in value, the histogram and fitted normal suggest that the data do not follow the normal distribution. In addition, there appears to be an outlier on the right side of the distribution.

Using the Analysis of Variance (ANOVA), there appeared to be no statistical difference between the group means of Apoptotic Index determined on H&E of the various histological subtypes ($p = 0.84$) or the various age/race groups ($p = 0.66$). There were very few White or Asian paediatric cases for analysis. The box and whisker plots illustrate how close the median values are in comparison to the spread around each median (See Figures 14 & 15).

Similar results were obtained using ApopTag i.e. Using the ANOVA, there appeared to be no statistical difference between the group means of Apoptotic Index for the various subtypes ($p = 0.33$) or the various age/race groups ($p = 0.56$) (see Figures 16 & 17).

TABLE 15 (Continued)

WHITE ADULT GROUP (>15 YEARS) (CONTINUED)

Age/Race	Sex	LMP1	Subtype	Stage	Rx	Default	Outcome	Refractory/Relapse
White adult	Male	Positive	MC	2	Chemo complete	-	Lost to follow up	Unknown
White adult	Female	Negative	NS	2	Chemo complete	-	Alive	-
White adult	Male	Positive	NS	3	Chemo complete	-	Lost to follow up	Unknown
White adult	Male	Negative	NS	2	Chemo complete	-	Alive	-

SUBTYPES:

MC - Mixed Cellularity
 NS - Nodular Sclerosis
 LD - Lymphocyte Depleted

TREATMENT:

DXT - Radiotherapy
 ABMT - Autologous Bone Marrow Transplant

TABLE 15 (Continued)
WHITE ADULT GROUP (>15 YEARS)

Age/Race	Sex	LMP1	Subtype	Stage	Rx	Default	Outcome	Refractory/Relapse
White adult	Female	Negative	MC	3	Chemo incomplete	-	Dead unknown cause	Unknown
White adult	Male	Negative	NS	3	Chemo complete	-	Lost to follow up	Refractory & Relapsed
White adult	Female	Positive	MC	1	Chemo, DXT	-	Died unknown cause	Refractory & Relapsed
White adult	Female	Positive	MC	1/2	Chemo complete	-	Alive	-
White adult	Male	Negative	MC	4	Chemo, DXT	-	Alive	Refractory & Relapsed
White adult	Male	Negative	NS	1/2	Chemo complete	-	Alive	-
White adult	Female	Negative	NS	3	Chemo complete	-	Alive	-
White adult	Male	Positive	NS	2	Chemo, SCR	-	Alive	Refractory & Relapsed
White adult	Male	Negative	MC	2	Chemo complete	-	Dead unknown cause	-
White adult	Male	Negative	MC	3	Chemo complete	-	Alive	-

SUBTYPES:

MC - Mixed Cellularity
 NS - Nodular Sclerosis
 LD - Lymphocyte Depleted

TREATMENT:

DXT - Radiotherapy
 ABMT - Autologous Bone Marrow Transplant

TABLE 15 (Continued)
BLACK ADULT GROUP (>15 YEARS) (CONTINUED)

Age/Race	Sex	LMP1	Subtype	Stage	Rx	Default	Outcome	Refractory/Relapse
Black adult	Female	Negative	NS	2	Chemo complete	-	Alive	Refractory or relapsed
Black adult	Male	Negative	NS	3	Chemo incomplete	-	Dead unknown cause	Unknown
Black adult	Male	Negative	NS	2	Chemo complete	Defaulted	Alive	Unknown
Black adult	Female	Positive	NS	3	Chemo complete	-	Alive	-
Black adult	Male	Positive	MC	4	Chemo incomplete	Defaulted	Lost to follow up	Unknown
Black adult	Female	Positive	MC	4	Chemo	-	Lost to follow up	Unknown
Black adult	Female	Negative	NS	2	Chemo ongoing	-	Alive	Refractory or relapsed
Black adult	Male	Positive	MC		Chemo incomplete	Defaulted	Lost to follow up	Unknown
Black adult	Female	Positive	MC	5	Chemo, DXT	Defaulted	Alive	Refractory or relapsed
Black adult	Female	Negative	MC	2	Chemo ongoing	Defaulted	Alive	-
Black adult	Female	Negative	NS	3	Chemo complete	-	Alive	-

SUBTYPES:

MC - Mixed Cellularity
 NS - Nodular Sclerosis
 LD - Lymphocyte Depleted

TREATMENT:

DXT - Radiotherapy
 ABMT - Autologous Bone Marrow Transplant

TABLE 15 (Continued)
BLACK ADULT GROUP (> 15 YEARS)

Age/Race	Sex	LMP1	Subtype	Stage	Rx	Default	Outcome	Refractory/Relapse
Black adult	Female	Negative	NS	2	Chemo	Defaulted	Dead - unknown cause	Refractory or relapsed
Black adult	Male	Negative	NS	2	Chemo incomplete	Refused Rx	Dead unknown cause	Refractory or relapsed
Black adult	Male	Positive	MC	4	Chemo incomplete	Defaulted	Lost to follow up	Unknown
Black adult	Male	Positive	MC	3	Chemo incomplete	Defaulted	Lost to follow up	Unknown
Black adult	Male	Positive	MC	2	Chemo incomplete	Defaulted	Lost to follow up	Unknown
Black adult	Male	Negative	NS	2	Chemo, DXT, SCR	Defaulted	Lost to follow up	Unknown
Black adult	Male	Negative	NS	4	Chemo incomplete	Defaulted	Lost to follow up	Unknown
Black adult	Female	Negative	NS	4	Chemo incomplete	Defaulted	Lost to follow up	Unknown
Black adult	Male	Positive	NS	1/2	Chemo complete	Defaulted	Alive	Refractory or relapsed
Black adult	Male	Positive	NS	2	Chemo complete	-	Lost to follow up	Unknown
Black adult	Male	Negative	NS	2	Chemo complete	Defaulted	Lost to follow up	Unknown

SUBTYPES:

MC - Mixed Cellularity
 NS - Nodular Sclerosis
 LD - Lymphocyte Depleted

TREATMENT:

DXT - Radiotherapy
 ABMT - Autologous Bone Marrow Transplant

TABLE 15 (Continued)

WHITE PAEDIATRIC GROUP (≤ 15 YEARS)

Age/Race	Sex	LMP1	Subtype	Stage	Rx	Default	Outcome	Refractory/Relapse
White paediatric	Male	Positive	MC	2	Chemo complete	-	Alive	-
White paediatric	Male	Negative	MC	2	Chemo & DXT	-	Dead - unrelated	-

ASIAN PAEDIATRIC GROUP (≤ 15 YEARS)

Age/Race	Sex	LMP1	Subtype	Stage	Rx	Default	Outcome	Refractory/Relapse
Asian paediatric	Male	Positive	NS	3	Chemo	-	Alive	-

SUBTYPES:

MC - Mixed Cellularity
 NS - Nodular Sclerosis
 LD - Lymphocyte Depleted

TREATMENT:

DXT - Radiotherapy
 ABMT - Autologous Bone Marrow Transplant

TABLE 17: DISTRIBUTION OF HISTOLOGICAL SUBTYPE ACCORDING TO AGE/RACE FOR THE CASES IN THE EPIDEMIOLOGY GROUP (n=175)

Histological subtype	Black Adults (n=43)	White Adults (n=71)	Asian Adult (n=1)	Unknown race Adult (n=1)	White Paediatric (n=14)	Black Paediatric (n=40)	Asian Paediatric (n=5)
Mixed cellularity	13	23	-	-	4	22	3
Nodular sclerosis	25	38	1	-	10	13	2
Lymphocyte depleted	-	1	-	-	-	2	-
Lymphocyte predominant	1	1	-	-	-	1	-
No lymph node biopsy	4	2	-	1	-	2	-
Unclassifiable	-	3	-	-	-	-	-
Unable to find	-	3	-	-	-	-	-

No lymph node biopsy - Indicates that the diagnosis was made on some other tissue.

TABLE 16: DISTRIBUTION OF AGE/RACE, AVERAGE AGE AND MALE:FEMALE RATIO IN THE EPIDEMIOLOGY GROUP (n = 175)

Age/Race	Black Adults	White Adults	Asian Adult	Black Paediatric	White Paediatric	Asian Paediatric
Number	43	71	1	40	14	5
Average Age	32yrs 7 m	41yrs 6m	58yrs	8yrs 6m	9yrs 11m	10yrs 7m
Male:Female Ratio	1,04:1	1,03:1	.	3,4:1	1:1	0,6:1

One adult male of unstated race, aged 29 years is not included in the table.

The 1 Asian case was a male of 58 years with nodular sclerosis HD. In one case the race of the patient was not obtainable, he was a male with no lymph node biopsy. Age 29 years.

Details of the adult age distribution are summarised in Table 19.

There were 116 Adult patients.

43 of these were Black adults with an average age of 32 years and 7 months and a male:female ratio of 1.04:1.

Their histological subtype distribution was as follows:

Mixed Cellularity	= 13.
Nodular Sclerosis	= 25.
Lymphocyte Predominant	= 1.
No lymph node biopsy	= 4.

71 were white patients with an average age of 41 years and 6 months and a male:female ratio of 1.03:1.

Distribution of histological subtype:

Mixed Cellularity	= 23.
Nodular Sclerosis	= 38.
Lymphocyte Depleted	= 1.
Lymphocyte Predominant	= 1.
No lymph node biopsy	= 2.
Unclassifiable	= 3.
Unable to find	= 3.

The distribution of subtypes were as follows:

Black children (n=40)

Mixed Cellularity = 22.

Nodular Sclerosis = 13.

Lymphocyte Depleted = 2.

Lymphocyte Predominant = 1.

No lymph node biopsy = 2 i.e. diagnosis was made on some other tissue.

White children (n=14)

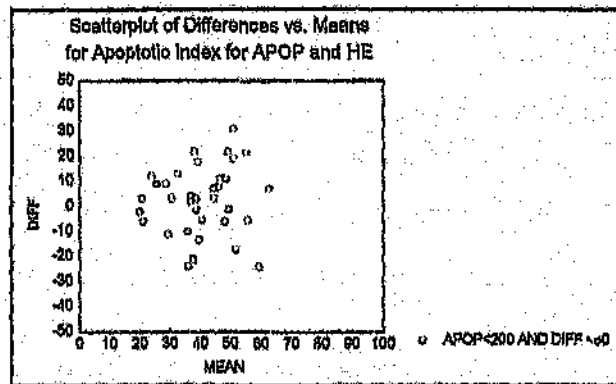
Mixed Cellularity = 4.

Nodular Sclerosis = 10.

Asian children (n=5)

Mixed Cellularity = 3.

Nodular Sclerosis = 2.

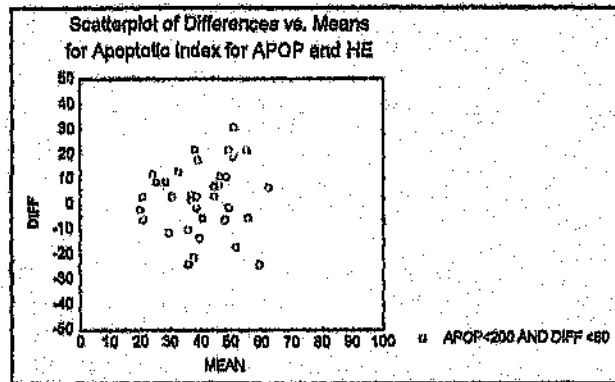


**FIGURE 23: SCATTER PLOT OF DIFFERENCE VS MEAN FOR APOPTOTIC INDEX
FOR APOPTAG AND H & E**

4.3 EPIDEMIOLOGY

Analysis of the data available on the 175 patients with a diagnosis of HD summarised in Tables 16 & 17.

There were 59 paediatric cases (Defined as ≤ 15 years). 40 of these were Black children with an average age of 8 years and 6 months and a male:female ratio of 3.4:1. 14 were White children with an average age of 9 years and 11 months and male:female ratio 1:1. 5 were Asian children average age 10 years and 7 months and male:female ratio 0.6:1. Detailed age distribution is summarised in Table 18.



**FIGURE 23: SCATTER PLOT OF DIFFERENCE VS MEAN FOR APOPTOTIC INDEX
FOR APOB AND H & E**

4.3 EPIDEMIOLOGY

Analysis of the data available on the 175 patients with a diagnosis of HD summarised in Tables 16 & 17.

There were 59 paediatric cases (Defined as ≤ 15 years), 40 of these were Black children with an average age of 8 years and 6 months and a male:female ratio of 3.4:1. 14 were White children with an average age of 9 years and 11 months and male:female ratio 1:1. 5 were Asian children average age 10 years and 7 months and male:female ratio 0.6:1. Detailed age distribution is summarised in Table 18.

There appears to be very poor agreement between the two methods used to assess Apoptotic Index. This is depicted in the scatter plot of Apoptotic Index established on ApopTag vs Apoptotic Index established on H&E - (see Figure 22), and corroborated on the scatter plot of the Difference vs the Mean of the two methods (see Figure 23). The scatter of the difference between Apoptotic Index on H&E and ApopTag appears to be narrower at smaller values of the mean than at large values. The mean difference was 3.67 and the standard deviation was 15.34.

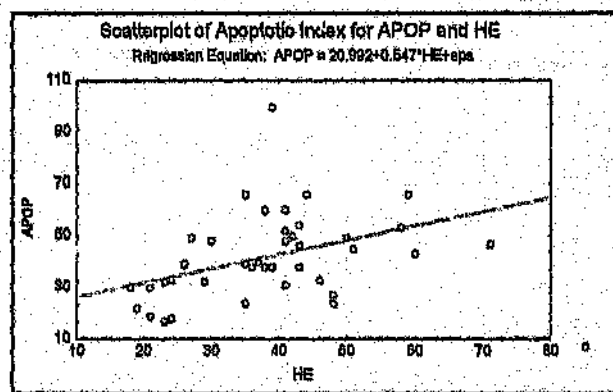


FIGURE 22: SCATTER PLOT OF APOPTOTIC INDEX FOR APOPTAG AND H & E

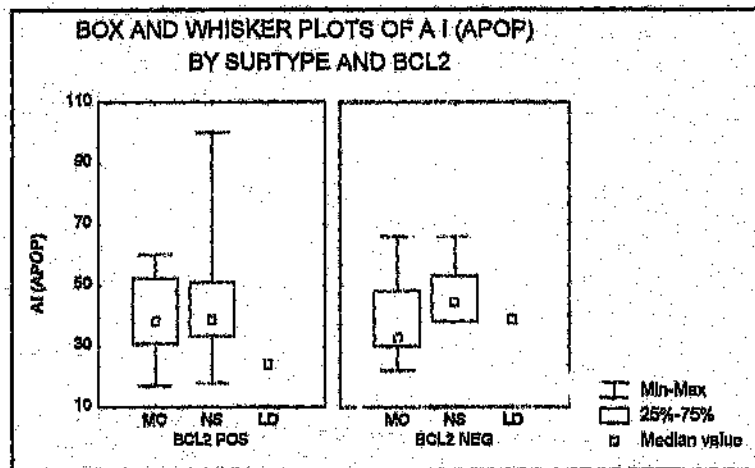


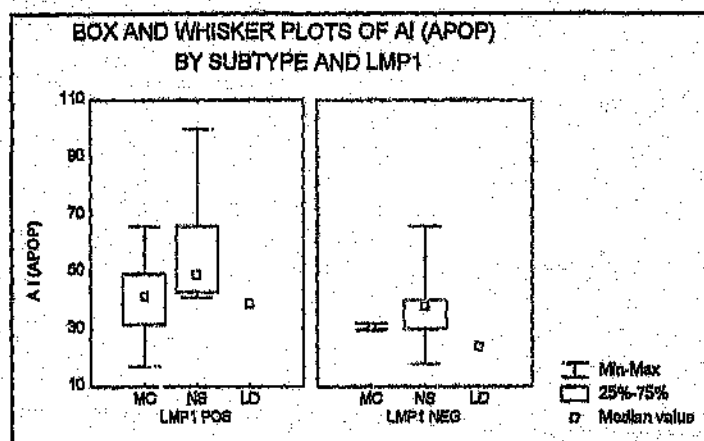
FIGURE 21: BOX AND WHISKER PLOTS OF APOPTOTIC INDEX ON APOPTAG BY SUBTYPE AND BCL-2

MC = Mixed Cellularity
 LD = Lymphocyte Depleted

NS = Nodular Sclerosis

The two-way ANOVA of Apoptotic Index (on ApopTag) by Subtype and Bcl-2 gave a p-value of 0.73.

The ANOVA of Apoptotic Index (on ApopTag) by Bcl-2 gave a p-value of 0.45.



**FIGURE 20: BOX AND WHISKER PLOTS OF APOPTOTIC INDEX ON APOPTAG
BY SUBTYPE AND LMP-1**

MC = Mixed Cellularity
LD = Lymphocyte Depleted

NS = Nodular Sclerosis

The two-way ANOVA of Apoptotic Index (on ApopTag) by Subtype and LMP-1 gave a p-value of 0.73.

The ANOVA of Apoptotic Index (on ApopTag) by LMP-1 gave a p-value of 0.07.

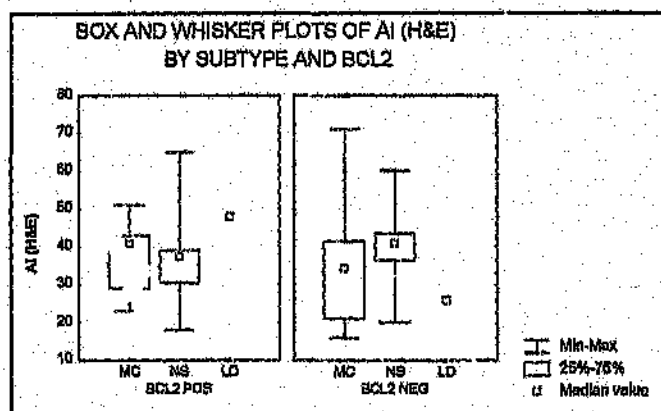


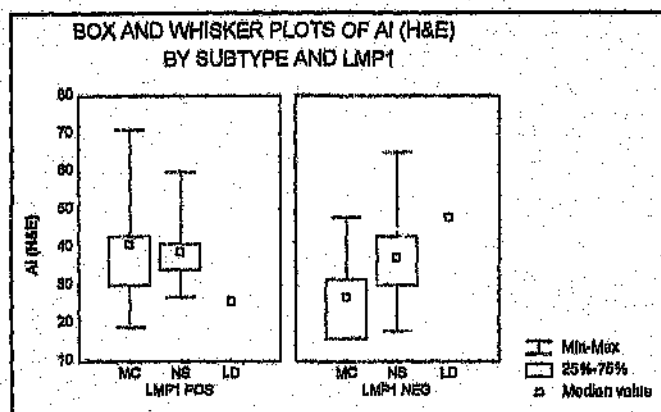
FIGURE 19: BOX AND WHISKER PLOTS OF APOPTOTIC INDEX ON H & E BY SUBTYPE AND BCL-2

MC = Mixed Cellularity
LD = Lymphocyte Depleted

NS = Nodular Sclerosis

The two-way ANOVA of Apoptotic Index (on H & E) by Subtype and Bcl-2 gave a p-value of 0.22.

The ANOVA of Apoptotic Index (on H & E) by Bcl-2 gave a p-value of 0.19.



**FIGURE 18: BOX AND WHISKER PLOTS OF APOPTOTIC INDEX ON H & E
BY SUBTYPE AND LMP-1**

MC = Mixed Cellularity
 LD = Lymphocyte Depleted

NS = Nodular Sclerosis

The two-way ANOVA of Apoptotic Index (on H & E) by Subtype and LMP-1 gives a p-value of 0.13.

The ANOVA of Apoptotic Index (on H & E) by LMP-1 gave a p-value of 0.58.

Nevertheless, there is evidence to suggest a role for LMP-1 in the pathogenesis of EBV positive HD as it is strongly expressed in concentrations that would be toxic to a normal cell. In addition, LMP-1 has been identified as being essential for immortalisation of B cells (in combination with other latent proteins), can transform human keratinocytes, and is known to have transforming potential in rat fibroblasts. Evidence suggests that, at least in HD, LMP-1 is not acting through up regulation of bcl-2. A seemingly neglected article, (Mosialos *et al*, 1995) describes the interaction of LMP-1 with Tumour Necrosis Factor Receptor associated Factors (TRAFs). LMP-1 interacts with a novel human protein LAP-1 as well as an EBV induced human protein EB16. Both these proteins interact with the p80 Tumour Necrosis Factor Receptor, and LAP-1 also binds CD40 and the Lymphotoxin β receptor. LAP-1 is homologous to a murine protein (TRAF 2) which is implicated in growth signalling from the p80 Tumour Necrosis Factor Receptor. It is hypothesized that normally, binding of Tumour Necrosis Factor Receptor causes cross linking of the extracellular domains of the Tumour Necrosis Factor Receptor, leading to aggregation of intracellular domains with their associated TRAFs, resulting in signal transduction. As LMP-1 molecules normally form a complex at the plasma membrane, this may lead to aggregation of LMP-1 associated LAP-1 and EB16 resulting in constitutive signal transduction and increased proliferation. In addition, competition between LMP-1 and p60 Tumour Necrosis Factor Receptor binding to LAP-1, may block induction of apoptosis usually mediated by that receptor.

EBV recombinants deleted for portions of the LMP-1 carboxy terminal domain, including the amino acids which interact with LAP-1 are incapable of transforming B Lymphocytes.

HD (Schiaffer *et al*, 1995). As BHRF-1 (an EBV gene which shares extensive homology with bcl-2) is expressed in the lytic rather than latent phase of EBV infection, this is also unlikely to contribute to tumorigenicity.

Another possible viral target could be p53, which functions as a cell-cycle checkpoint control, leading to either growth arrest in the G1/S phase of the cell or culminating in apoptosis. It has been reported that EBNA 5 which is required for EBV induced B cell transformation, can form a complex with p53 and the retinoblastoma protein, possibly impairing their tumour suppressor function (Szekely *et al*, 1993). However, like BHRF-1, EBNA 5 is not characteristically expressed in EBV positive HD, and is therefore unlikely to play a role in the pathogenesis of HD. A study from Natal (Bickhoo; Chetty, 1997) has showed that 39 of 43 EBV positive cases were negative for Retinoblastoma protein, suggesting that EBV may cause inactivation of the latter through another mechanism.

It has also been proposed that the growth of malignant cells and their interaction with other reactive cells, are mediated through cytokines. Production of several cytokines has been detected in Hodgkin's and Non-Hodgkin's Lymphoma. (Hsu *et al*, 1993).

In 1996, Klein *et al* demonstrated that most of the Burkitt lymphoma cell lines infected by wild type EBV or the EBV strain B95-8, produced IL8, IL10, TNF α , TNF β , in contrast to EBV negative lines or to cell lines infected with the non-transforming EBV strain P3HR1. Although results obtained from cell lines should probably be treated with reserve, further investigations along these lines may implicate viral induced cytokines in the pathogenesis of HD.

for EBERs (Weiss; Chang, 1992; Lauritzen *et al*, 1994) and is thought to have contributed to the high rates of EBV positivity obtained with PCR.

5.4 THE ROLE OF EBV IN HD

In 1991, a paper published in *Cell* using DNA transfection showed that LMP-1 could protect human B cells from apoptosis by up regulating the expression of bcl-2. (Henderson *et al*, 1991). This finding led to speculation that this process may play a role in the pathogenesis of EBV positive HD. Although 34/61 (55.7%) of the cases in our series showed bcl-2 expression in the Reed-Sternberg/Mononuclear variant cells, no positive association with LMP-1 expression was demonstrated. This lack of interaction between LMP-1 and bcl-2 has been reported by other authors. (Armstrong *et al*, 1992b; Jiwa *et al*, 1993; Jiwa *et al*, 1995).

In the present series 15/23 (65%) of Black adult cases 11/22 (50%) of White adult cases, and 8/16 (50%) paediatric cases were bcl-2 positive in Reed-Sternberg/Mononuclear variant cells. Nodular sclerosis subtype showed a higher rate of positivity when compared to mixed cellularity subtype (62.5% vs 48%).

One of the two cases of lymphocyte depleted subtype was bcl-2 positive.

It therefore, seems that despite the strong evidence for an association between EBV and HD, the exact pathogenetic role of the virus remains elusive.

It has been suggested that EBV may interact with bcl-2 homologues such as bcl-x. However, bcl-x expression is not specific to EBV positive HD, but is also noted in EBV negative cases of

Small numbers, variation in subtype (known to affect prognosis), and differences in clinical stage at presentation make accurate comparison of outcome in LMP-1 positive and negative cases difficult to predict in our series.

In a recent editorial (Oudejans *et al*, 1997) state that a group of 80 patients with nodular sclerosis or mixed cellularity HD, patients with EBV positive disease had a shorter progression free survival time. However, no differences were observed in overall survival time. Whether these patients were paediatric or adult is not specified. Similar results were observed by Vestlev *et al*, (1992). In contrast, the Cape Town study suggested that EBB positivity confers a more favourable outcome in childhood HD. Hence, the influence of LMP-1 on prognosis of HD remains to be resolved.

5.3 COMPARISON OF EBER vs LMP 1 - TECHNICAL CONSIDERATIONS

Contrary to other reports, *in situ* hybridisation for detection of EBERs, was less sensitive than immunohistochemical detection of LMP-1. 8/32 LMP-1 positive cases, were negative for EBERs on 2 separate occasions. The reasons for this are probably related to fixation/processing of the tissue.

Since *in-situ* hybridisation was time consuming and technically demanding, it is therefore suggested that immunohistochemical detection of LMP-1 would be a practical option for routine diagnostic use in the laboratory. A few (3) of the cases that were positive with LMP-1 and EBERs in the Reed-Sternberg/Mononuclear variant cells, also showed positivity in occasional background small lymphocytes. Similar findings have been reported with the *in-situ* hybridisation

A major shortcoming of the present study, was that there were insufficient White paediatric cases for comparison with Black paediatric cases. As most Black children in South Africa show EBV seroconversion at an early age, (Macdougall *et al.*, 1993), they might have been expected to show a significantly higher prevalence of EBV positivity in HD, than White children who may only be exposed to EBV in early adulthood. Such data would obviously need to be controlled for histological subtype, as the increased incidence of mixed cellularity subtype within the Black paediatric group would also increase the rate of EBV positivity.

Similarly differences may have been expected between White and Black adult cases of HD. In our series, Black adult cases showed a somewhat higher prevalence of EBV positivity as compared with White adult cases (47.8% vs 36.3%). This was primarily due to different prevalence of EBV positivity within the mixed cellularity subtype (87.5% vs 54.5%). However, the nodular sclerosis subtype showed similar rates of positivity among Black and White adult cases (26.6% vs 20%).

A recent report from Natal showed somewhat higher prevalence for EBV positivity in HD. 43 of 71 (60.6%) cases of HD were shown to be EBV positive using immunohistochemistry for LMP-1 and *in-situ* hybridisation for EBERs (Bickhoo; Chetty, 1997). This included 5/9 (55.5%) cases in the <15 year age group.

Comparisons of EBV positivity in adult cases of HD from Algeria and France using *in-situ* hybridisation for EBB DNA and RNA, showed 72% (n=25) and 33% (n=21) positivity respectively. Positivity was more frequent in mixed cellularity than other subtypes. A study of a Mexican adult population showed 35/50 (70%) of HD, to be EBV positive (Quintanilla-Martinez *et al.*, 1995), with all histological subtypes being associated.

There appears therefore to be fairly strong evidence supporting a role for EBV in HD in the paediatric age group, particularly <10 years. Whether this is subject to geographical or ethnic variation, will require further investigation.

TABLE 20: GLOBAL COMPARISON OF HD VS EBV IN PAEDIATRIC AGE GROUP

Country	Technique	Age	EBV Positive	Reference
UK, Brazil, Saudi Arabia	ISH for EBVIR, IHC for LMP-1 or Southern blot	1-4yrs 5-9yrs 9-15yrs	6/7 (85.7%) 18/20 (90%) 14/28 (50%)	Armstrong <i>et al</i> , 1993
USA Honduras	ISH for EBVIR, IHC for LMP-1	<9yrs 10-14yrs <9yrs 10-14yrs	4/7 (57%) 5/18 (27.7%) 8/8 (100%) 3/3 (100%)	Ambinder <i>et al</i> , 1993
Peru	ISH for EBVIR, IHC for LMP-1	<15yrs	18/20 (90%)	Chang <i>et al</i> , 1993
Konya	IHC for LMP-1	<16yrs	53/53 (100%)	Weinreb <i>et al</i> , 1996a
Malaysia	ISH for EBVIR	<15yrs	14/15 (93%)	Pelt <i>et al</i> , 1997
USA	IHC for LMP-1	0-4yrs 5-9yrs 10-15yrs	3/3 (100%) 8/13 (61.5%) 6/28 (21.4%)	Andriko <i>et al</i> , 1997
Natal	ISH for EBVIR, IHC for LMP-1	<10yrs 11-15yrs	3/4 (75%) 2/5 (40%)	Biekhov, Chetty 1997
Capo Town	ISH for EBVIR, IHC for LMP-1	<15yrs	32/47 (68%)	Sinclair-Smith <i>et al</i> , 1997
Hong Kong (Chinese population)	ISH for EBVIR	<15yrs	4/5 (80%)	Chan <i>et al</i> , 1995
China	ISH for EBVIR, IHC for LMP-1	<12yrs	4/4 (100%)	Zhou <i>et al</i> , 1993
UK	IHC for LMP-1	<5yrs 5-10yrs 10-15yrs	2/5 (40%) 12/27 (44%) 23/42 (48%)	Weinreb <i>et al</i> , 1992
UK Greece Jordan United Arab Emirates Iran Egypt South Africa Australia Costa Rica Kenya	ISH for EBVIR, IHC for LMP-1	2-14yrs 2-14yrs 3-11yrs 4-12yrs 3-12yrs 3-15yrs 5-14yrs 4-14yrs 2-13yrs 2-15yrs	38/75 (50%) 20/22 (90%) 8/16 (50%) 6/10 (60%) 7/8 (88%) 7/14 (50%) 9/18 (50%) 11/16 (70%) 34/42 (81%) 56/56 (100%)	Weinreb <i>et al</i> , 1996b

Other studies have suggested that paediatric cases of HD from developing countries have a much higher incidence of EBV positivity than figures quoted for the USA and Europe. A study comparing EBV positivity in paediatric HD in the United States and Honduras, showed 11/11 (100%) of the Honduras cases to be EBV positive vs 9/25 (36%) from the United States (Ambinder *et al*, 1993). 8/11 and 4/9 of these children from Honduras and USA respectively were ≤ 9 years of age. Recent work from Kenya (Weinreb *et al*, 1996a) quotes 100% EBV positivity in childhood cases of HD. A report from Peru of a largely paediatric group quotes EBV positivity in 94% of cases (Chang *et al*, 1993).

Investigators from the United States have now published findings of 38.6% EBV positivity in 44 paediatric cases of HD. These showed a striking age distribution: 3/3 cases LMP-1 positive in ≤ 4 years age group, 8/13 (61.5%) LMP-1 positive in the 5-9 year group; and 21.4% LMP-1 positive in the 10-15 year old group (Andriko *et al*, 1997) (See Table 20).

It is of interest that Weinreb *et al* (1996b), quote an EBV positive HD prevalence of 50% for South Africa in the 5-14 year age group. A recent report from Natal showed only 5/9 (55%) of cases of HD in the <15 year age group to be positive (Bickhoo; Chetty, 1997).

A report from Cape Town also showed a somewhat lower prevalence of EBB in childhood HD than was noted in our series (Sinclair *et al*, 1997). 32/47 (68%) of cases of paediatric HD were EBB positive, with a racial distribution of: 18/24 (75%) Mixed Race, 10/15 (67%) Black, 4/8 (50%) White. Similarly to our findings there was a trend for the mixed cellularity cases to be positive (4/5) when compared with the nodular sclerosis (28/42).

Data from the South African National Cancer Registry in 1988 (Sitas, 1992) in fact suggests that the overall frequency of HD is lower in Blacks of all ages, than in Whites. This may however, represent underreporting of cases, particularly from rural areas, where access to health care is limited.

Hospital based registries and studies from other African countries, including Zambia and Zimbabwe, suggest that the Type I epidemiological pattern predominates in these countries. (Emmanuel; Gelfand, 1975; Naik; Bhagwandeon, 1976).

5.2 PREVALENCE OF EBV IN HODGKIN'S DISEASE AT BARAGWANATH AND JOHANNESBURG GENERAL HOSPITALS

In our series the overall prevalence of EBV positivity assessed by immunohistochemical detection of LMP-1 in HD (52%) and its association with histological subtype (77.7% mixed cellularity subtype EBV positive, 31% nodular sclerosis subtype EBV positive) is similar to that reported for Europe and North America.

The prevalence of EBV was striking in the paediatric subgroup (10/13 black, 2/2 white and 1/1 Asian paediatric cases were EBV positive - 81% overall). Except for one child, all were <10 years of age, supporting Armstrong's findings that HD in this age group was particularly likely to be EBV positive. (Armstrong, *et al*, 1993). This latter study included 55 cases of paediatric HD from the United Kingdom, Brazil and Saudi Arabia and showed no significant difference in EBV positivity by country.

Although incidence rates cannot be calculated from this information the occurrence of HD and predominance of mixed cellularity subtype among black paediatric patients, suggests that this group may fit the Type 1 epidemiological pattern frequently noted in developing countries (viz: high rates in children and predominance of histological subtypes associated with poor prognosis) (Jarrett, 1992). It is difficult to comment whether the Black adult group comply to the expected low rate of HD in older age groups, usually noted in this pattern. Certainly within our groups these appear to be a significant number of patients (21/43) representing the 15-29 years age group which is not expected in a Type 1 epidemiological pattern.

Although no comment is possible regarding the incidence rate, white paediatric cases are reported, but these differ from the Black paediatric group in being predominantly of the nodular sclerosis subtype. 25/71 White adult patients fall into the 15-29 year age group. Although Whites are traditionally regarded as a higher socio-economic group in South Africa, our limited analysis does not show that the White patient group to fitting the classic Type III epidemiological pattern associated with developed countries (viz young adult peak, favourable prognostic histological subtype, with a second peak in older age groups). Again, this may represent bias in population groups presenting to government hospitals or may reflect bias introduced by only looking at a small portion of the total patient group.

There were insufficient Asian cases to comment on epidemiological patterns.

5.0 DISCUSSION

5.1 EPIDEMIOLOGY

Our data from 2 centres, suggests that the average age of Black ($n = 40$) and White ($n=14$) paediatric patients with HD was very similar (8 years and 6 months vs 9 years and 11 months). The few Asian children in the study ($n=5$), had a slightly older average age of 10 years and 7 months. Mixed cellularity subtype was more common amongst Black children than White children (55% vs 28.5%), whilst nodular sclerosis HD was the predominant subtype noted in White children (71.4% vs 32.5% in the Black paediatric population). The Black paediatric group conformed to the expected increase in male:female ratio, while the White paediatric group showed a 1:1 ratio. The latter is probably related to the predominance of nodular sclerosis subtype in this group.

In the adult group, both black and white patients showed the expected 1:1 male:female ratio, and nodular sclerosis HD was the commonest histological subtype in both groups (58% in blacks, 53.5% in whites). White patients tended to present at an older average age than Black patients (41 years and 6 months vs 32 years and 7 months).

This data obviously only represents a proportion of the HD seen in South Africa. In addition, lack of access to health care, recognition of oncology units as referral centres, particular subpopulations presenting to government hospitals rather than private oncology units etc, introduce bias into the data.

**TABLE 19: AGE DISTRIBUTION OF ADULT PATIENTS DIAGNOSED AS HAVING
HODGKIN'S DISEASE AT THE BARAGWANATH AND
JOHANNESBURG GENERAL HOSPITALS FROM JANUARY 1989 TO
DECEMBER 1993**

NUMBER OF PATIENTS		
Age	Black Ethnic Group	White Ethnic Group
15-20yr	4	11
20-24yr	6	6
25-29yr	12	8
30-34yr	7	10
35-39yr	4	3
40-44yr	2	5
45-49yr	3	6
50-54yr	2	4
55-59yr	1	1
60-64yr	1	3
65-69yr	1	4
70-74yr	-	2
≥75yr	-	6
TOTAL	43	71

TABLE 18: AGE DISTRIBUTION OF PAEDIATRIC PATIENTS DIAGNOSED AS HAVING HODGKIN'S DISEASE AT THE BARAGWANATH AND JOHANNESBURG GENERAL HOSPITALS FROM JANUARY 1989 TO DECEMBER 1993

NUMBER OF PATIENTS			
Age	Black Ethnic Group	White Ethnic Group	Asian Ethnic Group
0-1yr 11m	-	-	-
2-2yr 11m	2	1	-
3-3yr 11m	-	1	-
4-4yr 11m	-	1	-
5-5yr 11m	8	-	1
6-6yr 11m	3	1	-
7-7yr 11m	5	1	1
8-8yr 11m	3	-	-
9-9yr 11m	5	1	-
10-10yr 11m	3	1	-
11-11yr 11m	1	1	-
12-12y 11m	4	1	-
13-13y 11m	3	2	3
14-15yr 11m	3	3	-
TOTAL	40	14	5

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6.0 CONCLUSION:

This study of 61 patients with HD at the Baragwanath and Johannesburg Hospitals has shown the following:

The prevalence of EBV positive HD, using immunohistochemical detection of LMP-1 and *in situ* hybridisation for detection of EBERs is 52%, which is similar to figures quoted for Europe and North America.

- EBV positivity shows a strong association with mixed cellularity subtype.
- Children ≤ 10 years of age show a particularly high prevalence of EBV positive HD.
- There does not appear to be any interaction between LMP-1 and bcl-2 expression.

Although the actual incidence of HD was not determined, the Black paediatric population appear to approximate the Type 1 pattern noted in developing countries. The White population show some features of the type III Epidemiological pattern of developed countries, but is probably an intermediate pattern.

In this study, there is very poor agreement between morphological assessment of Apoptotic Index on H & E and immunohistochemical detection with ApopTag (Oncor USA).

Both methods showed no statistically significant difference in Apoptotic Index between LMP-1 positive and LMP-1 negative cases. This may suggest that LMP-1 is acting by increasing proliferation rather than inhibiting apoptosis, but could equally be attributed to the small number of cases. An unsatisfactory absence of a statistically significant difference in Apoptotic Index between bcl-2 positive and bcl-2 negative cases, was also noted on both methods, and is probably related to small case numbers.

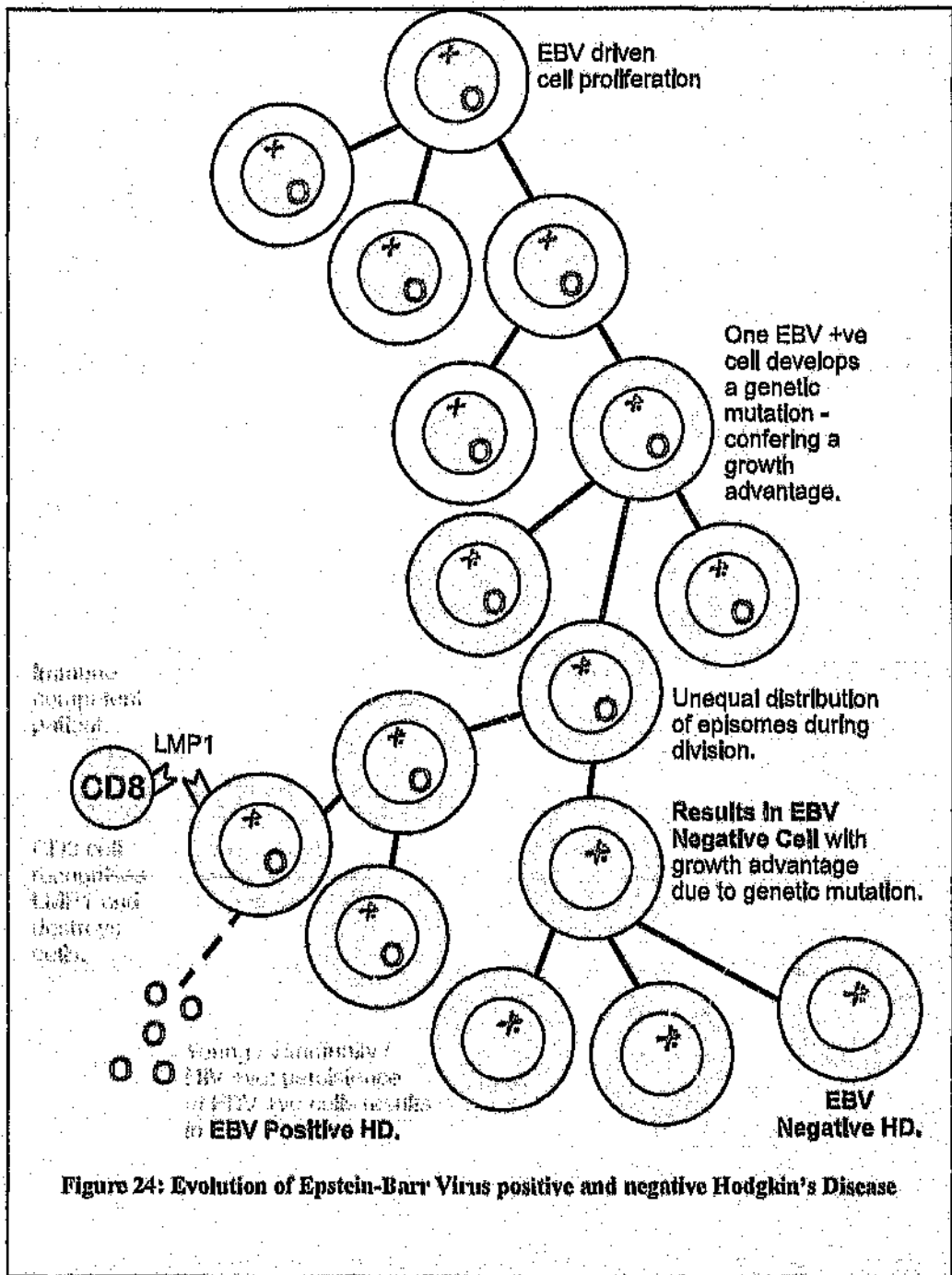
Although on morphological assessment mummified cells appear to be more prominent in certain histological subtypes, again neither method, showed any significant difference in Apoptotic Index between histological subtype.

5.5 APOPTOSIS:

Although historically "malignancy" has been associated with increased cell proliferation, the role of anti-apoptotic events in tumourigenesis is becoming increasingly recognised. Speculation regarding the role of EBV in HD has tended to concentrate on its anti-apoptotic characteristics rather than its more obvious proliferative effects.

Investigators from Cape Town (Close *et al*, 1994) examined apoptosis, using ApopTag, proliferation indices and expression of p53 and bcl-2 in Reed-Sternberg/Mononuclear variant cells. They found a slight inverse correlation between Apoptotic Index and proliferation indices, and identified a subset of cases with a high percentage of cycling cells, relatively low Apoptotic Index and expression of p53 and/or bcl-2. These findings were not related to EBV LMP-1 or EBER expression. This study showed great inter-case variability in Apoptotic Index. This was confirmed in the present study; which also showed poor agreement between the two methods used to assess Apoptotic Index viz morphological assessment of mummified cells on H & E, and ApopTag (ONCOR, USA). This obviously raises questions regarding our ability to recognise apoptotic cells on H & E, and whether mummified cells are truly apoptotic.

The results showed no obvious trends but appeared to be random (i.e. Apoptotic Index was not consistently higher or lower in one or other method). Although care was taken in trying to assess the same fields on each method (especially as they shared a common denominator as ascertained on H & E), this may not have been exact, and in view of the enormous inter-area variability regarding mummified cells, could have been a major contributing factor to the poor agreement between methods.



(similar to Burkitt's Lymphoma), rather than being the "final event" as it appears to be in Post transplant B-cell lymphoproliferative disorders.

Several authors interpret the findings of both EBV positive and EBV negative cases of HD, as support for MacMahon's "Multiple Aetiology Hypothesis" viz that HD is a grouping of at least 3 entities probably with quite distinct aetiologies.

Another scenario may also be plausible: it is possible that all cases of HD are initially associated with EBV. EBV would cause initial cell proliferation, but eventually the cells would develop another mutation which would confer on it a growth advantage independent of the presence of EBV. It is also possible (although it has not been described) that during cell division, unequal sharing of EBV episomes, may render such a cell EBV negative while still retaining its growth advantage, giving rise to apparently EBV negative HD.

The above process would still retain some EBV positive malignant cells: in immuno-competent individuals, LMP-1 expressed by these cells would be recognised as a target by cytotoxic T cells and removed. However, in children, and individuals with impaired immunity (age or nutrition related, or possibly related to other co-existing infections) the above events would be less efficient, leading to a higher incidence of EBV positive HD in children, HIV positive patients and developing countries (see Figure 24).

If racial differences are found to exist this could be possibly be ascribed to different HLA types favouring different viral epitopes.

alternatively that single base mutations occurring in a promotor/enhancer region could affect LMP-1 expression/effects in the absence of the 30bp deletion (eg by interfering with phosphorylation sites). These mutations could also act by rendering the strain non-immunogenic.

It is not only the pathogenetic role of EBV in HD that continues to frustrate investigators worldwide. Explanations (and often consensus) on the epidemiological patterns of EBV positivity also remain elusive. Overall, it appears that Europe, North America, and now South Africa show prevalences of EBV positivity in HD of ~40-50%, with a strong association with mixed cellularity subtype.

Adults in developing countries (including Mexico and Algeria) appear to have a higher prevalence of EBV positive HD, with at least one study showing strong associations with all histologic subtypes.

There seems to be emerging consensus that paediatric HD, particularly in the ≤ 10 year age group, has a strong association with EBV. Some studies suggest that this prevalence is higher in developing than developed countries.

The lower prevalence of adult EBV positive HD in developed countries, as well as the weak association (~30%) between EBV positivity and nodular sclerosis subtype, which is found predominantly in young adults in these countries, argues strongly against the "Delayed Exposure Hypothesis" that development of HD is related to late infection with EBV.

It is possible that EBV's role in HD is part of a multistep process of malignant transformation

The role of LMP-1 in the cell is ambiguous. Although it is responsible for B-cell transformation, its expression on the surface of the cell also makes it a target for cytotoxic T cell leading to cell destruction. It is interesting that at least two of the proposed pathogenetic roles for EBV in HD probably represent viral attempts to avoid destruction by the immune system viz, the above possible anti-apoptotic activity, and the production of an Interleukin-10 homologue and TNF β which inhibit cytotoxic T cells.

A naturally occurring variant of LMP-1 has been described in some cases of EBV positive HD (Sandvej *et al*, 1994; Bellas *et al*, 1996). This variant is the result of a 30bp deletion in the carboxy terminal end of the LMP-1 gene. It has been described in "ordinary HD", and in one study, showed a particularly high prevalence in HIV associated HD (Bellas *et al*, 1996). The deletion appears to be associated with histologically aggressive behaviour (as implied by a high frequency of neoplastic cells). Rather than affecting interactions with the Tumour Necrosis Factor Receptor pathways, this deletion occurs in a region of LMP-1 required for rapid protein turnover and may act to prolong the half- life of the protein, leading to accumulation within the cell.

This EBV strain (CAO-BNLF1) was initially identified in the nude mouse-propagated Chinese Nasopharyngeal Carcinoma CAO cell line. The CAO-BNLF-1 gene was shown to have a 30bp deletion and 7 single base mutations, and according to tumorigenicity studies on SCID and nude mice, was more tumorigenic than the LMP-1 protein encoded by the B95.8 EBV strain. It should be noted however, that the 30bp deletion and 6 of 7 single base mutations were found in 3/9 of tonsils of acute infectious mononucleosis in Denmark and therefore also occur in non-malignant states. Sandvej *et al* (1994) suggest that the 30bp deletion and single base mutations may arise independently and that the single base mutations could modify the effects of the deletion, or

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