THE ROLE OF EPSTEIN-BARR VIRUS IN THE PATHOGENESIS OF LYMPHOCYTIC INTERSTITIAL PNEUMONIA

by

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ABSTRACT

Epstein-Barr Virus (EBV) genome has been demonstrated in lung tissues of patients with lymphocytic interstitial pneumonia (LIP). Recent *in vitro* studies have shown that the EBV immortalizes and transforms cells by upregulation of the cellular proto-oncogene *bcl-2* via the viral latent membrane protein LMP1. The purpose of the studies described in this thesis was to determine whether the presence of EBV LMP1 and over-expression of *bcl-2* occurs in LIP. Immunohistochemistry was employed to detect EBV LMP1 and *bcl-2* in formalin-fixed, paraffin-embedded lung sections from patients with LIP ($n=14$), idiopathic pulmonary fibrosis (IPF, $n=9$) and autopsy cases (AC, $n=9$) without lung disease. EBV density, defined by the number of LMP1 positive cells per unit area of lung tissue, was used to estimate the level of LMP1 expression. The results showed increased LMP1 expression in lung tissue from patients with LIP ($p<0.05$). *bcl-2* expression was assessed by a grading system based on cellularity (Grade 1, 2 and 3) and intensity of immunostaining (Grade 0, 1, 2, and 3). *bcl-2* staining was greatest in LIP ($p<0.05$), and IPF showed more staining than AC ($p<0.05$). We conclude that EBV is more prevalent and LMP1 expression was increased in LIP associated with an upregulated *bcl-2* expression. These results support the hypothesis that EBV infection is important in the pathogenesis of LIP and *bcl-2* may play a role towards lymphomagenesis of LIP.

TABLE OF CONTENTS

LIST OF FIGURES.

LIST OF TABLES.

LIST OF ABBREVIATIONS.

AIDS	Acquired immunodeficiency syndrome
bcl-2	B-cell leukemia 2 gene
CR2	Complement receptor 2
EBERs	Epstein-Barr Virus encoded RNAs
EBNA	Epstein-Barr nuclear antigens
EBV	Epstein-Barr virus
HIV	Human Immunodeficiency virus
HSV	Herpes Simplex virus
LIP	Lymphocytic interstitial pneumonia
LMP	Latent membrane protein
LPD	Lymphoproliferative disorder
TR	terminal repeats
ZEBRA	Z Epstein-Barr virus replication activator

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DEDICATION

To:

My Parents,

Florence and John,

who kept on believing in me.

CHAPTER 1 GENERAL INTRODUCTION

1.1 Lymphocytic Interstitial Pneumonia.

Lymphocytic interstitial pneumonia (LIP) was first described by Carrington and Liebow in 1966 as an interstitial lung disease characterized by diffuse cellular infiltrations within the interstitium, expanding into the alveolar spaces (1). The infiltrates are predominated by a mixture of lymphocytes, plasma cells, mononuclear elements and reticulo-endothelial cells (1,2). As the lymphocytes infiltrate and proliferate, the structure of the lung changes and pulmonary function is compromised (3).

Prior to the beginning of the AIDS[1] epidemic in the 1980's, LIP was a rare condition usually associated with diseases thought to have an autoimmune basis. The most common presenting symptoms of LIP are progressive dyspnea and cough developing in a patient already diagnosed with Sjogren's syndrome or other autoimmune disorder (4,5), and more recently, human immunodeficiency virus (HIV) infection (6-9). The course of LIP is quite unpredictable: in some patients, the lesions remain stable following corticosteroid therapy, while in others, the lung function deteriorates progressively with end-stage fibrosis and eventually honeycomb lung (2,10). Presently, LIP is considered as an AIDS-defining illness for HIV-infected children (6-9,11).

Despite having been identified 3 decades ago, the etiology and pathogenesis of LIP is still unknown (2,10,12). Currently, the two most widely held proposals for

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[1] Acquired Immunodeficiency Syndrome

the pathogenesis of LIP are viral infections and autoimmune phenomena: the former, because of higher titers of Epstein-Barr virus (EBV)-related antibodies in LIP patients compared to non-LIP controls (7,8) and the high frequency of HIV-infected children with LIP (6,13-15), and the latter, the association of LIP with autoimmune conditions such as Sjogren's syndrome (16,17).

1.2 LIP and Epstein-Barr Virus.

The EBV has been related to lymphoproliferative disorders (LPD) (18-22) as well as Sjogren's syndrome (16,17) and several studies have suggested that the EBV may play a role in the etiology of LIP. Andiman *et al* (23) demonstrated EBV DNA in eight out of ten lung biopsy specimens showing LIP by Southern blot hybridization. Significantly higher titers of EBV antibodies were demonstrated in patients with LIP associated with AIDS, compared to HIV-positive patients without LIP (7,8). In addition, EBV DNA was demonstrated by *in situ* hybridization in nine out of fourteen LIP patients compared to two out of ten patients with idiopathic pulmonary fibrosis (IPF) (24). These observations support an association between EBV and LIP but the pathogenetic mechanisms of the involvement have not been defined.

1.3 LIP as a lymphoproliferative disorder.

The observations made by Liebow and Carrington (1) that the lung lesions in LIP patients were mainly lymphocytes and plasma cells were suggestive of the B lymphoid origin in the infiltrates. A recent immunophenotypic study using a pan-B cell marker (L26) yielded conclusive evidence confirming this speculation (24). Many lung pathologists are now convinced that LIP is a low grade lymphoma that has the

potential to evolve into a more aggressive pulmonary and/or systemic lymphoma (2,12).

High level expression of the human B-cell leukemia 2 (*bcl-2*) gene has been documented in various hematopoietic neoplasms as well as LPD (25-27). Over-expression of *bcl-2* contributes to the mechanism of LPD by inhibition of programmed cell death and thereby decreases the frequency of cell death, resulting in an accumulation of cells (28). One might therefore speculate that *bcl-2* could play a role in the etio-pathogenesis of LIP. In addition, detectable levels of EBV proteins have been found in several AIDS-associated lymphomas and LPD (21,29,30).

1.4 Summary.

The interest in LIP has been renewed in recent years because it has been estimated that 30% - 50% of HIV-infected children will develop this pulmonary complication (31,32). With this escalating population of HIV-infected patients, further research is required to define the pathogenesis of this lymphoproliferative process. Since LIP is a LPD associated with the EBV, it is therefore plausible that the etiology and pathogenesis of LIP could revolve around these areas, namely, *bcl-2* activity, lymphoproliferation and EBV-associated malignancies. The current study may provide an increased understanding towards the etio-pathogenesis of LIP in terms of EBV persistence as well as the mechanism of *bcl-2* activity in deregulating programmed cell death in other types of cancer and lymphoproliferative disorders.

CHAPTER 2 EPSTEIN-BARR VIRUS.

2.1 Historical Background.

Denis Burkitt first described a peculiar lymphoma in African children in 1958 (33). This lymphoma frequently involved the jaws and had a geographical distribution across equatorial Africa similar to that of yellow fever. Epidemiological studies linked the prevalence of the lesion to temperature and rainfall and Burkitt speculated that the lymphoma might be of viral origin (34). However, the search for a putative agent was not successful until 1964 when two independent groups, Epstein and Barr (35) and Pulvertaft (36) simultaneously reported the establishment of continuous lymphoblastoid cell lines from tumor samples of Burkitt's lymphoma patients. They described a new herpes-like virus detected in these lymphoblastic cell lines using electron microscopy. The most interesting property of this virus, later named the Epstein-Barr virus (EBV) after its discoverers, M.A. Epstein and Y.M. Barr, was that infection of human B lymphocytes leads to immortalization and indefinite proliferation of these cells.

2.2 Classification, virus and genome structure.

EBV belongs to the subfamily gammaherpesviridae and genus *Lymphocryptovirus*. Lymphocryptoviruses are found only in old world primate species and are usually named by the species in which they are naturally endemic; an exception is the EBV which is the only human lymphocryptovirus (37). Two major distinguishing features of EBV are the restricted *in vitro* host range and the ability to infect and immortalize human B lymphocytes leading to perpetual growth (38).

Classically, infections with lymphocryptoviruses are restricted to primate B lymphocytes *in vitro*; however, lymphocryptoviruses can also infect T cells, epithelial cells, and smooth muscle cells *in vivo* (39,40).

In general, the physical structure of EBV is highly similar to other herpesviruses. EBV has a toroid-shaped protein core wrapped with a double stranded linear DNA. This DNA-protein complex is enclosed by the nucleocapsid which is an icosahedral protein shell made up of 162 capsomeres. The major EBV capsid proteins are 160, 47 and 28 kDa and are similar in size to those of the herpes simplex virus HSV-2 (41,42). The nucleocapsid is protected by a lipid bilayer outer envelope with external glycoprotein spikes. The most abundant glycoproteins are 350/220 and 152 kDa and differ in size from those of the HSV-1 (41,42). In addition, EBV also differs from other herpesviruses in that its outer envelope is predominated by the glycoprotein gp350/220 (43).

The genome structure and organization are similar among lymphocryptoviruses, and the viral proteins are antigenically related among its species. EBV was the first herpesvirus whose genome was completely cloned and sequenced (44-46). It consists of approximately 172,000 base pairs of double-stranded DNA which encode about 80 proteins. Like other herpesviruses, the linear DNA molecule is divided into 5 unique domains (U1 to U5) interspersed by 4 internal repeat domains (IR1-4) and flanked by 2 terminal repeat (TR) domains on either end (Figure 1). The functions of many of the replication genes have been inferred from their homology to those of the herpes simplex virus. As for the EBV latency genes, counterparts in other human

Figure 1. Genomic structure of the Epstein-Barr virus.

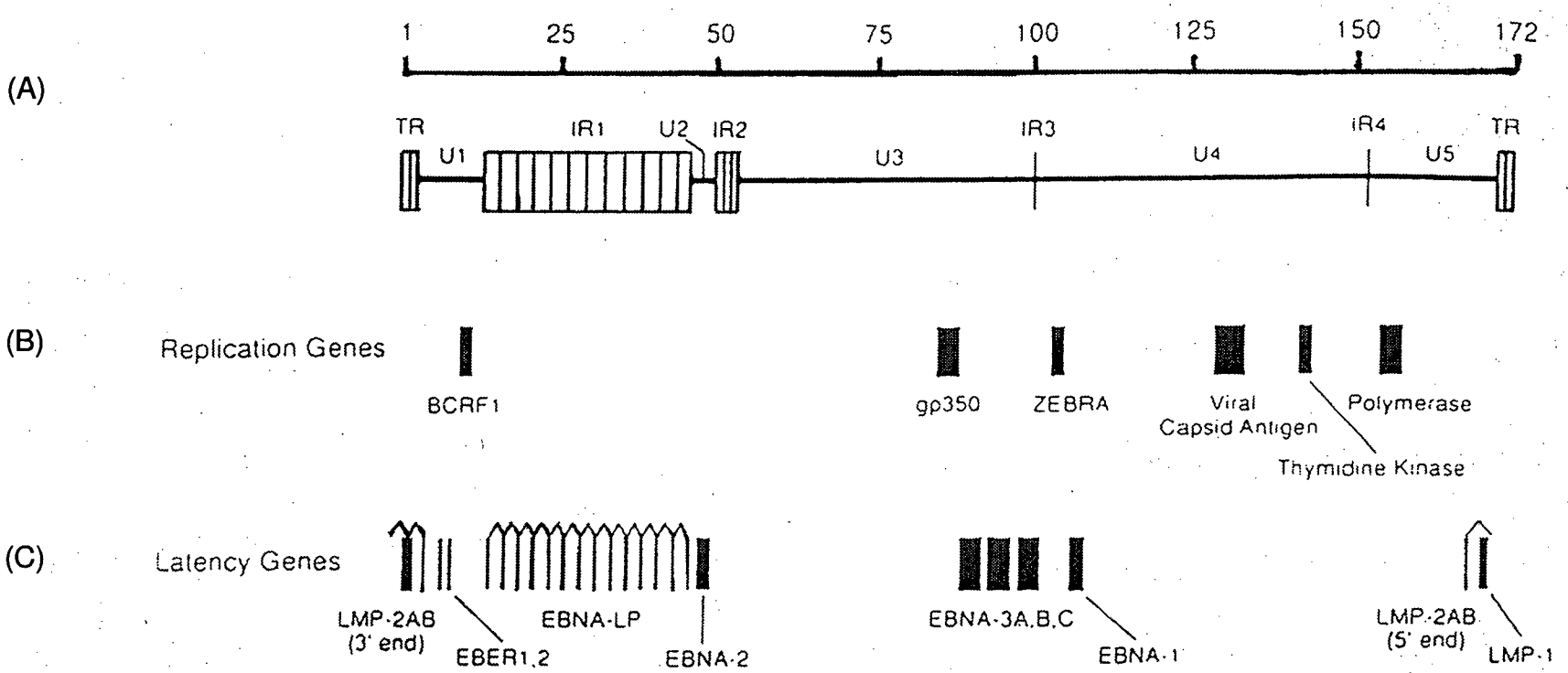
A) The EBV genome of approximately 172,000 base pairs of DNA is divided into 5 unique domains (U1 to U5) interspersed by 4 internal domains (IR1 to IR4) and flanked by 2 terminal repeat (TR) domains.

Black solid boxes denote coding regions of major replication an latency genes; regions where splicing occurs are denoted by (∧).

B) Among the replication genes, ZEBRA (Z Epstein-Barr virus replication activator) initiates the switch from latency to replication mode and transactivates early gene expression of EBV DNA polymerase, thymidine kinase BCRF1, followed by late expression of viral capsid antigen (VCA) and glycoprotein spikes gp350.

C) First viral proteins expressed during latency are EBNA (Epstein-Barr nuclear antigen)-LP (leader protein) and EBNA2 followed by other members of the EBNA family (EBNA1, 3A, 3B, and 3C), then the latent membrane protein family LMP1, LMP2A and LMP2B, and finally the EBER (Epstein-Barr virus encoded RNAs) family.

(Adapted from Cohen JI (54).)


(A)
1
25
50
75
100
125
150
172
TR
IR1
U2
IR2
IR3
IR4
TR
U1
U3
U4
U5
(B)
Replication Genes
BCRF1
gp350
ZEBRA
Viral
Capsid Antigen
Polymerase
Thymidine Kinase
(C)
Latency Genes
LMP-2AB
(3' end)
EBER1,2
EBNA-LP
EBNA-2
EBNA-3A,B,C
EBNA-1
LMP-2AB
(5' end)
LMP-1

herpesviruses have not been found and these may have arisen from cellular DNA (47). After the EBV enters the target cell, cellular transcription factors are responsible for the establishment of latency or the initiation of viral replication (48). The outcome of infection, which depends on the host immune response, determines the expression of different sets of viral genes. Infection of B-lymphocytes usually results in latent infection and transformation of the cell to a state of perpetual proliferation, a process known as immortalization (49). During latency, a limited set of EBV genes including those for the Epstein-Barr virus nuclear antigens (EBNAs), the latent membrane proteins (LMPs), and the Epstein-Barr virus encoded RNAs (EBERs) are expressed (50). On the other hand, if the viral replication cycle is initiated, viral genes encoding BCRF1, gp350, ZEBRA, viral capsid antigen, thymidine kinase, and polymerase are activated (50). The features of these EBV genes and the interaction of their products with host proteins will be discussed in the relevant sections.

2.3 Stages of Infection.

The usual route of EBV infection in humans is the oropharynx where viral replication is permissive in the oropharyngeal epithelium (51). Although it has been established that B lymphocytes are infected early in the course of primary infection as they traffic in close proximity to the oropharyngeal epithelium (37), the mechanism by which the virus traverses from the epithelial cell to the lymphocyte is not clearly understood. Diffusion of the virus across the epithelial basement membrane, B lymphocytes transit through the epithelium, or the action of an intermediary dendritic cell have all been suggested (52). The EBV does not normally replicate in B

lymphocytes but does establish a latent infection (37) which induces and maintains the B cells in a proliferative state. However, the virus is capable of replicating in B cells (53) and the frequency of this transition to a replicative phase increases with exposure to drugs that inhibit cellular macromolecular synthesis (55), DNA methylation (56), or with stimulation of protein kinase by phorbol ester (57).

2.3.1 Adsorption.

The EBV receptor is the type 2 complement receptor (CR2, now renamed CD21) expressed on the surface of B lymphocytes (58). The natural ligand for CD21 is C3d, a component of the common pathway in the complement cascade. CD21 is a member of the immunoglobulin superfamily with an extracellular amino-terminal domain, a transmembrane segment and a short carboxy-terminal cytoplasmic domain (59). The major EBV outer envelope glycoprotein gp350/220 is the CD21 ligand (60) and it binds to two or more of the repeats present in the amino-terminal domain of CD21 (61,62). CD21 is the only known B lymphocyte surface protein which binds to gp350/220 (60).

2.3.2 Penetration and Uncoating.

Penetration of the EBV is mediated by the viral glycoprotein gp350/220; contact between the B lymphocyte CD21 and the EBV gp350/220 results in patching and capping of CD21 (60). This in turn causes the enlargement and clumping of B lymphocytes without an increase in cellular RNA or DNA synthesis (60). The outcome of capping is endocytosis of EBV into smooth membrane vesicles, fusion of the viral envelope with the vesicle membrane and release of nucleocapsid into cytoplasm (60).

2.3.3 Establishment of Latency or Initiation of Replication.

The events between the release of the nucleocapsid-enclosed EBV DNA and its transport into the cell nucleus is not known (37). By analogy to other DNA viruses the cell microtubules and nuclear pore complex may mediate EBV capsid transport to the nucleus (63). The expression of the EBV receptor CD21 in a variety of cells permits efficient viral adsorption but this does not necessarily result in latent or lytic infection (64,65). This suggests that B lymphocyte or epithelial cell transcriptional factors may be necessary to establish the latent or lytic mode of viral infection (48). Since efficient viral replication could potentially kill the host, the strategy of the virus appears to be the establishment of lytic infection in the terminally-differentiated epithelial cells, with down-regulation of the lytic mode to latent infection in the B lymphocytes which allows the virus to persist (66,67).

2.3.4 Latent Infection.

Almost all EBV-infected cells contain multiple copies of covalently closed circular EBV episomes (68-70). After gaining entry into the B lymphocyte, the linear EBV genome circularizes (71,72). While it is recognized that active macromolecular synthesis is necessary for genome circularization, details of the mechanism are not known (52). From *in vitro* studies, only about 10% of the EBV coding capacity is utilized in B cells during latent infection; the other 90% is expressed when the immortalized cell begins to support the replicative phase of the EBV cycle (45). The latency genes include 2 small EBV encoded non-polyadenylated RNAs (EBER1 AND EBER2), 6 nuclear proteins (EBNA1, 2, 3A, 3B, 3C and LP) and 3 latent membrane

proteins (LMP1, 2A and 2B) (73,74) (Figure 1). The EBER genes are transcribed by host cell RNA pol III while the EBNA and LMP genes are transcribed by cell RNA pol II (75). The first viral proteins expressed during latency are EBNA LP and EBNA2, which reach stable levels 24-32 hours post infection (71). Expression of nuclear antigens are generated from alternate splicing of a long transcript (76). By 32 hours, all EBNAs and LMP1 are expressed and within 48 hours post infection, the EBNA proteins have reached their maximum levels (71). The EBER genes are expressed relatively late and reach substantial levels about 70 hours post infection (71).

2.3.4.1 Latent Infection Proteins.

For the purpose of this thesis (see section 4.1), the function and role of LMP1 will be emphasized while those of the other latent infection proteins will only be discussed briefly. The EBNA-LP is a nuclear protein that is highly phosphorylated and is associated with the nuclear matrix (77,78) during latency. The EBNA1 protein binds to the episomal origin of replication, oriP (79), and this protein-DNA binding activity is essential for maintenance of the EBV episome within the cell nucleus during latency (77,78). Transcription of EBNA2 leads to transactivation of cellular CD21, CD23 (80,81) and *c-fgr* (82) and viral latent membrane proteins LMP1 and LMP2 (83-85). CD23 is the low affinity receptor for Ig E and is involved in antigen presentation associated with major histocompatibility complex (MHC) II antigens. In addition, CD23 was recently shown to interact with CD21, the EBV receptor (86). *c-fgr* is a member of the *src* oncogene family and encodes a protein kinase important for B-cell growth regulation (54). The function of two other members the EBNA

family, EBNA 3A and 3B are not clear but EBNA 3C had been demonstrated to upregulate CD21 mRNA and protein (81).

2.3.4.2 Latent Membrane Protein 1 (LMP1).

In contrast to the other EBV latent genes, the LMP genes are transcribed in the opposite direction with respect to the other latent genes. As mentioned in the preceding section, the LMP1 promoter is upregulated by EBNA2 (83,84,87); however, LMP1 expression could also take place independently of EBNA2 in some Burkitt's lymphoma cells (88,89) and nasopharyngeal carcinoma tumors (85,90) following activation of lytic replication. The LMP1 transcript consists of 3 exons. From the primary amino acid sequence, an integral membrane protein with 3 domains is predicted: (a) a hydrophilic amino-terminus of 20 amino acids, (b) 6 markedly hydrophobic alpha-helical transmembrane segments of 20 amino acids each separated by 5 short reverse turns, and (c) a long carboxy-terminus of 180 amino acids (50,91). Although the LMP1 shares no sequence homology with other known proteins, its tertiary structure is similar to that of the rhodopsin family of cell surface receptors (92), certain ion channels (93,94) and some other integral membrane proteins, such as erythrocyte membrane band III or the *mas* oncogene (52). LMP1 forms cap-like structures in the plasma membrane of transformed cells and colocalizes with vimentin (91,95,96). Nascent LMP1 has a short half life of 2 to 5 hours; however, the half life of the cytoskeletal form of LMP1 is about 16 to 20 hours (95). These properties of LMP1 correlate with its ability to transform rodent cells (97) and LMP1 is the only protein produced during latent infection that is also associated with EBV lytic

replication (50). LMP1 is likely to be required for immortalization because of its effects on the growth of established cell lines *in vitro* and *in vivo* (49). LMP1-transformed rodent cells are capable of growth in media with reduced serum levels, grow in an anchorage-independent manner and are tumorigenic in nude mice (98-100). These observations indicate an oncogenic role for LMP1 (49).

Expression of LMP1 in lymphoblasts from EBV-negative Burkitt's lymphoma results in changes similar to those found in normal B cells when they are activated by EBV or mitogens. LMP1 could induce the lymphocytes from EBV-negative Burkitt's lymphoma to proliferate as clumps of cells with increased villous projections (101), increase their cell surface expression of CD23, CD39, CD40 and CD44 (102), and increase expression of vimentin (103) and cell adhesion molecules LFA1, ICAM1 and LFA3 (101). Induction of these cell adhesion molecules can bring about interaction between cells: binding of LFA1 to ICAM1 results in homotypic cellular adhesion which may explain the cell-cell aggregation or clumping seen when Burkitt's lymphoma cells proliferate due to EBV infection (49,104). In addition, upregulation of LFA3 brings about increased interaction between B cells and T cells since LFA3 is the ligand of the T cell antigen CD2 (104). LMP1 has also been shown to protect B lymphocytes from apoptosis through upregulation of the cellular proto-oncogene *bcl-2* (105,106), while LMP1 transfected cells seem to be more responsive to TGFβ (101).

LMP1 has been observed to alter the growth properties of established epithelial cells. It inhibits human epithelial cell differentiation (107) and transforms the RHEK-1 epithelial cell line from a keratinocyte-like morphology to that of a fibroblast (108).

Transgenic mice harboring the LMP1 transgene exhibit epidermal hyperplasia that eventually leads to chronic dermatitis (109). These results support a role for LMP1 in the EBV-induced transformation of the nasopharyngeal epithelium to an undifferentiated nasopharyngeal carcinoma. However, evidence from *in vivo* experiments in transgenic mice indicate that EBV transformation alone is not sufficient to convert the EBV-infected epithelium to nasopharyngeal carcinoma, indicating that additional factors are required for malignancy (107,109).

2.3.4.3 EBV Latency.

Presently, little is known about the interaction between EBV and the host cell that underlies EBV latency; however, the B cell compartment has been speculated as the site of EBV latency (76). This speculation was based on 2 lines of evidence : firstly, individuals receiving acyclovir therapy do not demonstrate a reduction of EBV-infected B cell pool despite elimination of EBV replication in epithelial cells (110), and secondly, patients who undergo total lymphoid irradiation, which destroys the lymphoid system but not epithelial tissues, were able to reverse the original EBV-carrier status (111). In order for EBV-infected B cells to maintain long term survival, these cells must be capable of evading immune recognition by EBV-specific cytotoxic T-lymphocytes, possibly by sustaining a passive latent form of EBV infection with minimal viral gene expression (76). Experimental evidence has demonstrated that EBV-carrying B cells can be readily triggered into the lytic cycle and if such signals were present in the lympho-epithelial environment, infectious virus could be effectively delivered from the recirculating B-cell compartment to permissive epithelial sites (76).

In some herpesviruses, such as HSV, there is a transitory period of viral replication before the virus enters into latency; EBV, however, directly enters into latency with no transitory period (73). Since EBV replication could lead to cell lysis and death of the host, the direct entry of EBV into the latency mode may be one of the reasons for the successful viral life-long persistence in the host.

2.3.5 Lytic Infection.

Although it has been presumed that LIP is associated with latent EBV infection, it is not known whether lytic EBV infection could occur in the lung tissue of LIP patients. Studies using lymphoblastoid cell lines transformed with EBV have shown that while the majority of the cells have latent infection, a few cells (<10%) are spontaneously activated to release virus (73). Lytic infection of EBV is usually studied by reactivation of EBV in latently infected B lymphocytes through the use of inducers such as phorbol esters (112) or superinfection (113). As with other herpesvirus infections, cells that are permissive for EBV replication undergo cytopathic changes such as margination of chromatin, inhibition of macromolecular synthesis, viral DNA synthesis and other related virion packaging activities (50). These inducers initiate expression of the viral BZLF1 gene, an immediate-early gene, to produce the ZEBRA protein. The function of ZEBRA is two-fold: (i) ZEBRA acts as a trigger switch to initiate viral replication in latently infected B cells and (ii) ZEBRA also upregulates the expression of other immediate-early genes and itself (114). It has been proposed that the ZEBRA polypeptide activates EBV immediate early gene expression by transcriptional activation through its DNA-binding capacity (115). The ZEBRA protein

contains a DNA binding region homologous to that of the *c-jun* oncogene and binds to AP-1 sites (116). The immediate early genes, early-antigen D complex and early-antigen R complex, in turn upregulate the expression of early gene products such as the viral DNA polymerase and thymidine kinase (114). Finally, the late genes representing the viral structural components such as the viral capsid antigen (VCA) and glycoprotein spikes gp350 are produced in preparation for packaging and release of infectious virions (114).

2.4 Summary.

Today, EBV is not only epidemiologically associated with Burkitt's lymphoma, anaplastic nasopharyngeal carcinoma (117) and infectious mononucleosis (118), but this ubiquitous herpesvirus has also been found in T-cell lymphoma (119), Hodgkin's disease (120) and lymphoproliferation in patients with acquired immunodeficiency (121). The increased number of human diseases associated with EBV not only reflects the advancement of research and diagnostic procedures in identifying viral genomes and gene products but also the success of the virus in maintaining itself in human cells. Although the mechanisms have yet to be elucidated, previous studies (7,8,23,24) have implicated the role of EBV in the etiology and pathogenesis of LIP. This thesis examined the role of the virus through expression of the LMP1 gene in lung biopsies from LIP patients.

CHAPTER 3 LYMPHOPROLIFERATIVE DISORDERS (LPD).

This chapter gives a brief introduction to lymphoproliferative disorders (LPD) to provide a basis for a discussion of the role of EBV in these disorders. The topic will be discussed within the context of proliferation versus programmed cell death and the oncogenes that may be involved.

Malignant neoplasms that arise from cells originating from a common pluripotent, primitive lymphoreticular stem cell are collectively known as LPD. LPD are classified according to the lineage of the affected cells and the specific point in the normal ontogenic process at which the cells were transformed, resulting in maturation arrest (122). In the human immune system, the repertoire of lymphocytes are derived from a hematopoietic stem cell via multistage differentiation into functional mature B and T lymphocytes. Hence LPD can be divided into the B-cell or T-cell series depending on the lineage of the affected cells. Common examples of B-cell LPD include follicular small (and large) cell lymphomas, chronic lymphocytic leukemia and Burkitt's lymphoma. LPD of the T-cell series, among many others, encompass lymphoblastic lymphoma, acute lymphoblastic leukemia and adult T cell leukemia/lymphoma.

3.1 Neoplasia: Proliferation versus prohibited programmed cell death.

Homeostasis in normal tissue is established when the balance between proliferation and death of its cells is at equilibrium (123). When this balance is violated, either due to uncontrolled growth or impaired cell death, neoplasia may result. Classically, neoplastic transformation of the immune system is due to

uncontrolled growth originating from a single clone of cells (122). On a molecular level, excessive proliferation is associated with 2 categories of proto-oncogenes: (i) growth and proliferation genes and (ii) tumor suppressor genes. Growth and proliferation genes, such as *myc*, *ras* and *abl*, promote proliferation by a "gain of function" mechanism affecting transcription factors or the signal transduction pathway. On the other hand, tumor suppressor genes as exemplified by p53 and the retinoblastoma gene product Rb, prevent aberrant cellular proliferation in their wild-type form. Tumor suppressor genes contribute to neoplasia by a "loss of function" mechanism in that abnormal tumor suppressor gene products fail to inhibit unwarranted growth and proliferation. Therefore, malfunction of these two categories of proto-oncogenes allows excessive proliferation leading to neoplasia.

The disruption of tissue/cell homeostasis has been revolutionized by a new concept in that cells are genetically programmed to die unless further signalling is initiated to block the intrinsic and autonomous suicide pathways (124,125). There are two forms of cell death: necrosis and apoptosis. Necrosis is usually the result of plasma membrane damage caused by disease or injury. The initial response is cellular swelling followed by an increase in density of mitochondria, flocculation of nuclear chromatin and decline in protein synthesis. At this point, the cellular response is reversible upon removal of stimulus; however, if the stimulus persists, irreversible changes occur with an increase of cytosolic Ca^{2+} levels. Eventually, cell death occurs when the plasma membrane ruptures to release cellular contents into the extracellular space leading to the initiation of an inflammatory reaction (126-128). Programmed

cell death, also known as apoptosis, is a directed and regulated form of cellular eradication whereby cells are removed without damage to the local environment (129). The sequential events of apoptosis include condensation of chromatin, nucleolar disintegration and cell shrinkage. Formation of apoptotic bodies composed of various components from ribosomes, organelles and nuclear material follows and the final stage of apoptosis is degradation of DNA into nucleosomal length fragments by endonucleases. A new category of oncogenes that regulate programmed cell death are associated with apoptosis. As a member of this novel category of oncogenes, *bcl-2* functions as an antidote to programmed cell death by the "gain of function" mechanism.

3.2 LPD and *bcl-2*.

The *bcl-2* gene was initially described in follicular lymphoma in which the t(14;18) translocation was a cytogenetic hallmark (130). Normally in humans, the *bcl-2* gene is located at 18q21; in follicular lymphoma, *bcl-2* is translocated to the immunoglobulin heavy chain locus (14q32) such that the *bcl-2* gene is under the strong influence of the Ig enhancer elements. This results in an over-expression of *bcl-2* mRNA and protein (131-133). The *bcl-2* gene encodes a 26 kD intracellular integral membrane protein associated with the mitochondria in lymphoid cells. Expression of *bcl-2* is not limited to lymphoid cells but is extended to cells and tissues which undergo apoptotic turnover (134). Expression of *bcl-2* has been demonstrated in lymphoid germinal centers; stem cells in the bone marrow; glandular epithelium of breast, thyroid and prostate tissue; and small crypt cells in both small and large

intestines (134). In its normal function, *bcl-2* relates to the generation and maintenance of memory B cells (135). Over-expression of *bcl-2* in cell lines has been shown to extend the survival of hematopoietic cell lines following growth factor deprivation and to interfere with programmed cell death. Gene transfer studies have demonstrated that *bcl-2* has unique biological properties that confer a prolonged survival advantage on both lymphoid cells and fibroblasts (136-138). These observations suggest that aberrant expression of the *bcl-2* protein is required in the development of a large proportion of human B-cell tumors. In addition, recent studies have shown that *bcl-2* may play a role in tissue morphogenesis (139) and growth factor-triggered signal transduction (140).

It has been frequently observed that in a variety of LPD such as B-cell non-Hodgkin's lymphoma, follicular lymphoma and diffuse B-cell lymphoma the chromosomal translocation of the *bcl-2* gene is present and the B cells display intense *bcl-2* staining (26,141). In a majority of these t(14,18) translocations, the translocation clusters within 2 regions: the major breakpoint region (mbr) (133,142) and the minor cluster region (mcr) (142,143) resulting in an intact *bcl-2* coding region so that the *bcl-2*/Ig heavy chain transcript encodes a normal *bcl-2* protein (144,145). However, recent studies demonstrate that high level *bcl-2* expression is also observed in both lymphoid and myeloid cell lines, with and without *bcl-2* gene rearrangement (25,26,146). These results not only indicate that *bcl-2* involves a wide spectrum of hematopoietic neoplasms but also that high level expression of *bcl-2* could be generated by mechanisms other than chromosomal translocation (25,26,146).

3.3 LPD and EBV.

EBV has been known as the causal agent of infectious mononucleosis, Burkitt's lymphoma and nasopharyngeal carcinoma. In addition, EBV is associated with a variety of LPD in patients with congenital, acquired or no apparent immunodeficiency. In the last few years, the advent of modern techniques with increased sensitivity and specificity led to an escalated rate of detection of EBV in human malignancies. Immunocompromised patients with LPD and AIDS are among the most frequently associated with EBV (21,29,121,147-151). In general, EBV-associated LPD range widely and include Hodgkin's disease (152-154), non-Hodgkin's lymphoma (151) and bone marrow transplant recipients who develop T-cell leukemia or chronic myelogenous leukemia (29,147). By PCR, conserved regions of the EBV genome encoding capsid protein and EBNA1 genes were amplified from biopsies of LPD patients (155). Expression of latent genes EBNA2 and LMP1 together with B lymphocyte adhesion molecules, ICAM1 and LFA3, have been demonstrated in lymphoproliferative lesions (29). Furthermore, evidence of DNA replication of EBV in the lytic cycle as well as EBV diffuse early antigen gene products occurred frequently in lymphomas and lymphoproliferative lesions (21).

As discussed in Chapter 2, EBV infection of B cells via the C3d complement receptor (CD21) results in the expression of viral antigens, polyclonal activation, induction of Ig secretion, continuous infection and "immortalization" of the cells. The primary result of the EBV infection is to cause proliferation of the infected lymphocytes. An important point to emphasize is that these immortalized cells are

capable of continuous growth in culture but are not malignant (i.e. do not cause tumors in nude mice and will not grow in soft agar). In most of the LPD, EBV acts as a predisposing factor among various factors and contributes to the pathogenesis of LPD via complex interactions with environmental and genetic elements (149). The role of EBV in neoplastic disorders remains obscure but its manifestation in related diseases is a reflection of the host's ability to control and suppress the proliferation of these infected B-cell clones (122).

In vitro studies with Burkitt's lymphoma cell lines have shown that expression of EBV latent genes not only activates B-cell proliferation but also mediates enhanced survival of these infected cells (106). EBV infected B cells upregulate cellular activation antigens (for example CD23, CD39 and CD70) and adhesion molecules (such as LFA1, ICAM1 and LFA3) in the same way as when normal B cells are stimulated by antigens or mitogens (156-158). Furthermore, the *bcl-2* gene is known to be activated after mitogenic stimulation of normal B and T cells (159). These observations led investigators to speculate a link between EBV latent protein expression, *bcl-2* gene activation and cell survival (105). Subsequent DNA transfection studies have indicated that EBV-infected B cells were protected from programmed cell death through the expression of *bcl-2* under the influence of a single EBV latent protein, LMP1 (105,160).

3.4 Summary.

In view of LIP as a LPD (section 1.3) together with supporting evidence of the association of EBV in LIP (6-8,23-24,161), one may question whether the

pathogenesis of LIP is due to the classical theory of profuse cellular proliferation or the prevention of programmed cell death. This thesis addresses this issue and the results will lead to important aspects of the pathogenesis of LIP and perhaps provide further insights towards the understanding of other LPD associated with EBV.

CHAPTER 4 EXPERIMENTAL PROTOCOL

The introductory chapters of this thesis provided the background information for the general hypothesis. An overview of LIP in the context of a LPD and its association with EBV was presented in Chapter 1. In Chapter 2, the biology of the EBV and its modes of infection were discussed. Chapter 3 described LPD and introduced the concept of apoptosis and its relationship with the proto-oncogene, *bcl-2*, and how this oncogene may provide an alternate route leading to neoplasia. As the clinical and pathological manifestations of LIP are consistent with a LPD, it is conceivable that abnormal function of *bcl-2* could play an important role in the pathogenesis of the lymphocyte proliferation in this condition. This possibility prompted the investigation into the EBV latent membrane protein, LMP1, and its possible role in regulation of the cellular gene, *bcl-2*.

4.1 General Hypothesis.

The general hypothesis of this investigation was:

That EBV plays a role in the pathogenesis of LIP via expression of the viral latent membrane protein, LMP1, which results in over-expression of the cellular *bcl-2* gene.

The purpose of the specific aims described below was to determine if there is an association between the presence of EBV LMP1 and the expression of *bcl-2* in LIP.

4.2 Strategy.

To test the relationship between EBV and LIP two approaches were used: (1) detection of the EBV LMP1 gene at the DNA level by the polymerase chain reaction (PCR) and (2) demonstration of the expression the viral LMP1 gene at the protein level

using immunohistochemistry. The EBV latent membrane protein, LMP1, was selected because of its essential role in cell transformation and its involvement in the upregulation of the host cellular protein, *bcl-2*, in *in vitro* systems. Since the *bcl-2* gene is expressed constitutively at low levels for normal physiological functions while high level expression is associated with neoplasia, it was necessary to establish an optimally low antibody concentration to distinguish cells that are aberrantly protected from programmed cell death.

4.3 Specific Aims.

There are 3 specific aims in this study:

1) To demonstrate the presence of the EBV in lung tissue of patient with LIP by PCR and compare these levels to those in control lungs.

2) To demonstrate and quantify the expression of the viral latent membrane protein, LMP1, by immunohistochemistry in lung tissue of LIP patients in comparison to those in the control groups.

3) To compare the expression of the cellular *bcl-2* proto-oncogene in lung tissues of LIP patients to that of control groups using immunohistochemistry.

4.4 Summary.

This study, an extension of the investigation of the role of EBV in LIP by Barberá *et al* (24), examined the general hypothesis using 2 major approaches: (1) PCR and (2) immunohistochemistry. These techniques were applied in the investigation of the etio-pathological role of the EBV at the DNA and protein levels, respectively.

CHAPTER 5 MATERIALS AND METHODS

5.1 Sample Information.

5.1.1 Patient Population - Clinical Evaluation.

The three groups of patients in this study were those with LIP (n=13) and two control groups: idiopathic pulmonary fibrosis (IPF, n=9) and autopsy cases (AC, n=9) of non-respiratory illnesses. The lung tissues were formalin-fixed and paraffin-embedded and were collected from our departmental files or obtained from other pathologists for the purpose of this study. The thirteen LIP cases and nine IPF cases have been used in a previous study (24). The IPF cases were included as subject controls in this study because patients with IPF have clinical characteristics similar to patients with LIP. Prior to the start of the experiments, the diagnoses of LIP and IPF were confirmed by two pathologists (Drs. J.C. Hogg and R.G. Hegele) based on clinical evaluations, radiographic and histological findings of the specimens. The nine autopsy cases with normal lung pathology were obtained from the Dept. of Pathology, St. Paul's Hospital. The causes of death include cardiac related illnesses (n=7), intracerebral hemorrhage (n=1) and trauma (n=1). There is no record of serologic studies for EBV or evidence of acute EBV infection in any of these patients. Patients in all three groups had similar age and sex distribution and this information is summarized in Table 1. Detailed information of the individual patients are listed in the Appendix.

Table 1. Summary of samples.

Group	n	Mean Age (yr ± SEM)	♂:♀
LIP	13	56.1 ± 9.0	4:9
IPF	9	59.7 ± 7.2	6:3
AC	9	64.0 ± 15.7	7:2

5.1.2 Cell Lines and Control Tissues.

Two human leukemia/lymphoma cell lines were used in this study. Namalwa cells (ATCC, Rockville, MD), which are derived from a latently EBV-infected African Burkitt's lymphoma cell line and have two copies of EBV DNA per cell (162), were used for extraction of DNA which served as control DNA for amplification of both HLA DQ*α* and EBV LMP1 genes. The Namalwa cells were also used as positive control for immunostaining by both anti-LMP1 and anti-*bcl-2* monoclonal antibodies (mAb). BJAB, a lymphoblastoid cell line that lacks the EBV genome (163), served as a negative control for the anti-LMP1 mAb. Both cell lines were cultured in 92.5% RPMI medium (Gibco, Gaithersburg, MD) with 7.5% fetal bovine serum (Hyclone, Logan, UT). Confluent cell cultures of these two lines were concentrated, resuspended in 1% low melting point agarose (Gibco) and embedded in paraffin. In addition to these control cell lines, two tissue controls were included: a tumor from a case of nasopharyngeal carcinoma (Mount St. Joseph's Hospital) and a lymph node from a

case of follicular lymphoma (Dept. of Pathology, St. Paul's Hospital) served as positive tissue controls for anti-LMP1 mAb and anti-*bcl-2* mAb, respectively.

5.2 Polymerase Chain Reaction (PCR).

5.2.1 Selection of Oligonucleotides.

The EBV LMP1 primer sequences were modified from those used by Chen *et al.* (164) in that both primers were extended by two bases at each end. The amplification of the EBV LMP1 gene spans the entire exon 1 and part of exon 2 to give a fragment of 501 bp. A region with a AT:GC ratio of 1:1 between the two primers was selected as an internal probe (25mer) to the amplified target of the EBV LMP1 gene. A conserved region of the HLA DQα gene was amplified using published sequences (165,166) and the flanking primers delimit a 242 bp product. The probe for detecting the amplified fragment of the HLA DQα gene is a 24-base sequence with approximately 50% GC content. The primers and internal probes are listed in Table 2. With the exception of the HLA DQα internal probe (Synthetic Genetics Inc., San Diego, Ca), all oligonucleotides were custom made by the DNA Synthesis Laboratory, University of Calgary.

Table 2. Sequences of oligonucleotides used in the PCR analysis.

Gene	Primer	N	Sequence (5'→ 3')
HLADQα	Forward	26	GTGCTGCAGGTGTAAACTTGTACCAG
	Reverse	28	CACGGATCCGGTAGCAGCGGTAGAGTTG
	Internal	24	TGGACCTGGAGAGGAAGGAGACTG
EBVLMP1	Forward	22	CCAGAAACACGCGTAACTCTCA
	Reverse	22	GGTACAATGCCTGTCCGTGCAA
	Internal	25	TCATCAGTAGGAGTAGACAAAGGCT

5.2.2 Purification of Primers.

The oligonucleotides were supplied by the manufacturer in a lyophilized, desalted form. Since all the oligonucleotides were less than 30 nucleotides long, a simple procedure using the C18 Sep-pak cartridge (Millipore Corporation, Bedford, MA) was used to remove non-nucleotidic contaminants (167). Each crude oligomer was dissolved in 0.5 M ammonium acetate and passed through an acetonitrile (Fisher Scientific, Vancouver, B.C.) rinsed C18 cartridge. The aqueous salts were eluted while the oligonucleotide was retained by adsorption on the column matrix. The column was flushed with distilled water before the oligonucleotide was eluted with 20% acetonitrile/water. The concentration of the column purified oligonucleotide was determined by measuring its optical density at 260 nm (A260) on a spectrophotometer (Perkin Elmer, Norwalk, CT). As 1 absorbance unit at 260 nm is equivalent to 37 μg/ml of single-stranded DNA, the volume of solution necessary to yield the desired amount of oligonucleotide could be calculated.

5.2.3 DNA Extraction.

Four 20 μm sections were cut on the "820" microtome (Spencer) using a fresh blade for each paraffin block to avoid carry-over contamination. DNA was extracted using the standard protocol (168). Briefly, the samples were deparaffinized using Histoclear™ (Diamed Laboratory Supplies Inc., Mississauga, Ont.), washed twice in 95% ethanol and left to dry in a vacuum desiccator. Proteinase K (Gibco) digestion was carried out overnight at 50°C. The aqueous phase was extracted by phenol/chloroform (1:1, BDH Inc., Vancouver, B.C.) three times, followed by two chloroform extractions and DNA precipitation in 7.5 M ammonium acetate (BDH Inc.) and 95% ethanol. The DNA pellet was resuspended in 100 μl of distilled water.

5.2.4 PCR Amplification.

The amplifications were performed on the GeneAmp PCR System 9600 (Perkin Elmer). Thirty microliters of each DNA sample were used for the amplification of the HLA DQα gene, and another 30 μl for the EBV LMP1 gene. For each preparation of extracted DNA, successful amplification of the HLA gene was required before the EBV PCR was performed. This ensured the analyses of PCR-grade DNA. DNA extracted from Namalwa cells was used as control for the optimization of PCR conditions for both HLA DQα[2] and EBV LMP1[3] genes. The cycling temperatures were set at 94°C

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[2]Amplification of the HLA DQα fragment was optimized in a total volume of 100 μl containing 10 mM Tris HCl (pH 8.4), 50 mM KCl, 2.5 mM $MgCl_2$, 0.2 mM of dATP, dCTP, dGTP, dTTP each, 0.5 μM of HLA DQα forward and reverse primer each, 2.5 units Taq DNA polymerase (Gibco) (final concentration).

[3]The EBV LMP1 PCR reaction mix was similar to that for amplification of HLA DQα except for the $MgCl_2$ concentration which was reduced to 1.5 mM and the EBV LMP1

for denaturation, 60°C for annealing and 72°C for extension over 50 cycles with 10s and 30s at each temperature for HLA DQα and EBV LMP1 amplification, respectively. The annealing temperatures and these time intervals were determined empirically for each primer set.

5.2.5 Agarose Gel Electrophoresis.

Twenty microliters of the PCR products together with 5 μl of loading buffer (15% Ficoll 400 (Sigma), 0.1 M Na_2 EDTA (pH 8, Fisher Scientific), 1% SDS (Fisher Scientific), and 0.25% xylene cyanol (Eastman Kodak Company, Rochester, NY)) were loaded into 6 mm wide wells in a 1.5% agarose gel (Gibco) containing 0.01% ethidium bromide (Sigma, St. Louis, MO). Electrophoresis in 1X TBE (90 mM Tris-HCl (pH 8, Sigma) 90 mM boric acid (BDH Inc.), 2 mM Na_2 EDTA (Fisher Scientific)) as running buffer was carried out at 150 V for 1 hour in a gel apparatus (Pharmacia LKB, Uppsala, Sweden). A photograph of each gel illuminated from below by a Fisher Biotech FBTI 816 UV transilluminator (312 nm) was taken through a Kodak #22 Wratten gelatin filter using a Polaroid MP-3 Land Camera with Polaroid film (Polaroid Corporation, Cambridge, MA). The amplified products were then transferred onto Hybond-N (Amersham, Arlington Heights, IL) membranes using the Southern blotting technique (Section 5.2.6).

5.2.6 Southern Transfer.

The agarose gels were immersed in a denaturing solution (1.5 M NaCl (BDH Inc.), 0.5 M NaOH (Fisher Scientific)) for 20 minutes with continuous gentle shaking

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primers were used in place of the HLA DQα primers.

at room temperature. The buffer was then replaced with neutralization buffer (1.5 M NaCl, 0.5 M Tris-HCl pH 7.2, 1 mM Na_2 EDTA) and gently agitated for another 20 minutes. Each gel was placed over 3 sheets of Whatman 3MM filter paper (Whatman International Ltd., Maldstone, UK) saturated with 20X SSC blotting buffer (3 M NaCl, 0.3 M $Na_3Citrate.2H_2O$, pH 7 (BDH Inc.)) which acted as a wick from the same blotting buffer. A piece of Hybond-N membrane was cut to the exact size of the gel and placed over the gel. Any air bubbles between the membrane and the gel were squeezed out using a glass pipette. Another 3 pieces of 3MM paper (cut to same size as the Hybond-N membrane) were placed on top of the Hybond-N membrane. A stack of absorbent paper towels was placed on top of the 3MM paper and a 0.75-1.0 kg weight was placed on top. Saran wrap™ was used to seal the borders of the gel to prevent direct transfer of buffer from the wick to the absorbent paper and to minimize evaporation during transfer. This set-up permitted the transfer of the DNA from the gel onto the membrane by capillary action for 12-18 hours. After blotting, the membrane was air-dried and the DNA on the membrane was UV-crosslinked at 312 nm for four minutes using a transilluminator (Fisher Biotech).

5.2.7 Labelling of Internal Oligonucleotide Probe.

Internal oligonucleotide probes were radiolabeled at the 5'end using the polynucleotide kinase reaction. Twenty pmole of oligonucleotide, 50 μCi γ^{32}P-ATP (3000 Ci/mole, 10 μCi/μl; Amersham) and 4.5 units of T4 polynucleotide kinase (Pharmacia LKB) in a total reaction mixture of 20 μl containing 0.05 M Tris-HCl pH 8.0, 0.01 M $MgCl_2$ (BDH Inc.), 100 μM Na_2 EDTA, 100 μM spermidine (Sigma) and

10 mM DTT (Sigma) was incubated at 37°C for 45 minutes. The reaction was stopped by heating at 65°C for 10 minutes. The labeled probe was purified by DEAE-cellulose chromatography in a 0.7 x 10 cm Biorad column containing 0.5 ml DEAE-cellulose (Sigma) equilibrated in TE (10 mM Tris-HCl pH 8.0, 1 mM Na_2 EDTA). Free phosphate ions and unincorporated nucleotides were eluted by TE and 0.2 M NaCl in TE, respectively. The labelled probe was eluted by 1 M NaCl in TE in 3 fractions of 0.5 ml each. A 5 μl aliquot of each fraction was analyzed in a Beckman LS 7500 Scintillation counter and the fraction with the highest number of counts was used as probe for filter hybridization.

5.2.8 Filter Hybridization and Autoradiography.

The Hybond-N filters with the DNA transferred from the agarose gels were first incubated at 65°C for a minimum of two hours in a sealed plastic bag containing the hybridization buffer (6X SSC; 5X Denhardt's solution (0.1% (w/v) Ficoll, 0.1% (w/v) polyvinylpyrrolidone (Sigma), 0.1% (w/v) BSA Pentax Fraction V (Sigma)); 0.5% SDS; 0.05 M sodium phosphate (pH 6.8, Fisher Scientific) and 20 μg/ml denatured salmon sperm DNA (Sigma)). A minimum volume of 80 μl of hybridization buffer/cm^2 of filter was used in the prehybridization stage and this volume was reduced to 40 μl/cm^2 when hybridizing with the labelled probe. One corner of the bag was cut, the excess buffer removed and the radiolabeled probe in fresh hybridization buffer was added. The bag was resealed and doubled-bagged to prevent leakage. The filters were incubated overnight at 65°C. After hybridization, the probe solution was removed, and the filters were washed in 6X SSC at room temperature for 5 minutes three

times, followed by another 30 minutes wash at 65°C. Washing was continued at 65°C until the background counts on the filter registered below 5 cps on the hand-held Geiger counter (Mini-Instruments Ltd., Essex, England).

The moist filters were mounted onto clear film, covered with Saran wrap and placed in Kodak Lanex screen-lined Kodak X-Omatic cassettes containing Curix RP-1 X-ray film (Agfa-Gevaert N.V., Belgium). The films were exposed either from 4 hours at room temperature or up to 72 hours at -70°C, depending on the strength of the radioactive signals, and then developed in a Kodak RPX-Omat Processor.

5.2.9 Densitometry.

The amount of human DNA extracted from the paraffin-embedded sections of lung tissue was too low to be detected by usual assays for DNA (Hoechst staining or absorbance at 260 nm). In order to estimate the amount of this DNA, the autoradiographic intensity of the HLA DQα PCR product from standard amounts of DNA extracted from Namalwa cells (7 pg to 5000 pg) was used to establish a linear relationship between the intensity of the band and the amount of human DNA present. The autoradiographic intensity of the HLA DQα PCR products from the lung samples was measured using the LKB Ultroscan XL Enhanced Laser Densitometer and the GelScan XL software program (Pharmacia LKB). These were compared to those of the standards in order to estimate the amount of human DNA suitable for PCR present in the extracted DNA samples. In the same way, a linear relationship between the intensity of the LMP1 PCR product and the amount of EBV DNA present was established using DNA from Namalwa cells (7 pg to 5000 pg). Likewise, the

autoradiographic intensity of EBV LMP1 PCR products from lung samples were compared to those of the standards to estimate the number of EBV genomes present. By using the estimated number of cells, based on HLA DQα PCR, as a denominator, the number of copies of EBV genomes per cell could be compared among the three patient groups.

5.3 Immunohistochemistry.

5.3.1 Preparation of Tissue Sections.

Microscopic slides were cleaned using detergent containing 2% Decon 75 (BDH Inc), rinsed in 100% ethanol, dried, soaked in 2% aminopropyltriethoxy-silane (Sigma) in acetone (Fisher Scientific) and washed twice in acetone. The paraffin-embedded specimens were cut into 5 μm sections using the "820" microtome as described in Section 5.2.3. Four sections from each block were used for staining with the two monoclonal antibodies and their respective controls. The sections were floated onto a 43°C water bath to remove the creases and then transferred onto the silane-coated slides that have been labelled appropriately. The slides were air-dried before use or stored at room temperature.

5.3.2 Optimization of Antibody Concentrations.

The two specific mAb, EBV anti-LMP1 and human anti-*bcl-2*, were purchased from Dakopatts (Glostrup, Denmark). Tris-buffered saline (TBS; 0.05 M Tris-HCl pH 7.6, 0.9% NaCl,) containing 5% bovine serum albumin was used as a diluent for both the mAb. As suggested by the suppliers, the anti-LMP1 mAb was diluted 1:50 while for the anti-*bcl-2* mAb, a dilution of 1:400 was determined empirically such that

lymphoid cells which normally express *bcl-2* would demonstrate little or no staining with the anti-*bcl-2* mAb, while cells exhibiting excessive expression of the *bcl-2* gene would remain positively stained.

5.3.3 Staining Procedures.

The standard protocol for alkaline phosphatase anti-alkaline phosphatase (APAAP) technique was employed for both antibodies (169). First, the lung sections on the silane-coated slides were deparaffinized using Histoclear™. The slides were then boiled in 10 mM citric acid (pH 6-6.3; BDH Inc) for 10 minutes. This pretreatment to unmask antigenic sites was suitable for the binding of both antibodies to formalin-fixed, paraffin-embedded tissue (170). After boiling, the slides were cooled to room temperature over 15-20 minutes and rinsed in distilled H_2O. The sections were incubated in 5% normal rabbit serum in TBS for 30 minutes to minimize non-specific staining. After this blocking step, the primary antibodies or the corresponding IgG_1 controls were applied to the tissue sections and allowed to incubate at room temperature for one hour. All antibody staining procedures were carried out in parallel with staining by mouse IgG_1 at a concentration comparable to that of the test antibody. The specificities of both the mAb were assessed by comparison of the test section to the corresponding control section stained with the mouse IgG_1. After one hour, the primary antibodies and the IgG_1 mAb were washed off using the same TBS buffer, three times at five-minute intervals. Excess buffer was blotted off and the slides were incubated for 20 minutes with rabbit anti-mouse immunoglobulin (1:20 dilution, Dakopatts). After washing and blotting as above, the

sections were incubated with the APAAP reagent (1:50 dilution, Dakopatts) in TBS for 20 minutes. During this incubation period, the New Fuchsin substrate[4] was prepared fresh for each staining experiment. The sections were placed in the substrate solution for 20 minutes after which the color reaction was stopped by rinsing the sections in TBS. The sections were counterstained with Mayer's hematoxylin before mounting them in organic solvent medium (Merck, Rahway, NJ).

5.3.4 Light Microscope Evaluation.

The slides were coded without knowledge of tissue identity prior to microscopic examination. The code was revealed only after all slides had been evaluated so that further analyses of the results could be made.

5.3.5 Estimation of EBV Density.

The cross-sectional area of each lung section was determined using the BioQuant BQ System IV software (R&M Biometrics Inc., Nashville, TN). This software program contains a feature which integrates a measured circumferential distance to calculate the enclosed area. The cross-sectional area (A) of the lung tissue section from each sample was obtained as the mean of the integrated areas from 3 measurements of the tissue outline. The total number of cells (n) stained by the anti-LMP1 mAb in each section was counted using a hand-held counter. In order to correct for the variation in cellularity in the different groups of specimens, the volume

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[4]The substrate solution was prepared in a final volume of 50 ml TBS (pH 7.6) containing 0.02% sodium nitrite (Sigma), 0.0001% New Fuchsin (Sigma), 1 mM levamisole (Sigma), and 25 mg napthol AB-BI phosphate (Sigma) dissolved in 300μl dimethyl formamide (BDH Inc).

fraction of tissue component (f) was estimated by the method of point counting (171). Point counting was carried out by superimposition of a 7x12 grid system of crosses over the microscopic image of tissue. This was done through a telescopic mirror attachment on the microscope which reflects the grid from the computer screen onto the image of the tissue in the microscope. The observer then scores the number of crosses superimposed on tissue, airspace and blood vessel. The volume fraction of tissue component (f) was calculated as the percentage of crosses superimposed on tissue over the total number of crosses counted. For each slide, point counting was performed on 5 randomly selected fields. The product of the mean area (A) and the volume fraction of tissue was used to estimate the volume fraction of tissue within the given area. The number of EBV infected cells per unit area of lung tissue, which will be referred to as EBV density, was then calculated according to the formula:

$$EBV\ Density = \frac{n}{f \times A}$$

where n is the number of LMP1 positive cells, f = volume fraction of lung corresponding to tissue and A = area of the slide taken up by lung tissue.

5.3.6 *bcl-2* Scoring System.

A semi-quantitative grading system of two histological features, cellularity and intensity of *bcl-2* staining, was developed. The degree of cellularity ranged from 1 to 3, based on an ascending order of the number of cells stained for *bcl-2*. The intensity of *bcl-2* staining was graded from 0 to 3, with grade 0 = little or no staining, grade

1 = weak staining, grade 2 = moderate staining, and grade 3 = intense staining. Sample photomicrographs depicting all the twelve combinations of cellularity and intensity were chosen randomly from all three patient groups. Using these photomicrographs (Figure 2) as standards, slides were evaluated using the 16X objective lens. Before examination, the lung section on each slide was divided into 10 segments of approximately equal size using a marker pen. A random field within each segment was selected for scoring and the 2 histological features were graded independently but concurrently. The sum of scores of the ten random fields in each slide was represented as the cumulative score for each of the histological features.

To assess the reproducibility of the *bcl-2* scoring system, the intra-observer and inter-observer variations were determined for each of the two histological features analyzed. The histological scores from two independent analyses of the same field were expressed as a 3 x 3 matrix for cellularity and a 4 x 4 matrix for intensity. Total agreement between the analyses (e.g. a score of "2" from observer/session A also being scored "2" from observer/session B) is expected to fall along the diagonal of the matrix. Since the *bcl-2* scoring system was designed to portray consistency of scoring between and within observers, the rows and columns were expected to be highly correlated. Therefore, the variation for each histological feature can be evaluated by calculating the ratio R_2/R_{2max} where R_2 is the Pearson coefficient of mean square contingency and R_{2max} is the maximal possible value of the Pearson $\chi 2$ coefficient if there is 100% agreement in the scores. The Pearson coefficient of mean square contingency (R_2) is a means of evaluating the dependence between rows and

columns in a matrix. The intra- and inter-observer variations were calculated for each histological feature using the following formula:

$$Variation = \frac{R_2}{R_{2max}} = \frac{\sqrt{\frac{T}{N+T}}}{\sqrt{\frac{Q-1}{Q}}}$$

where T = Pearson χ^2 statistic, N = number of observations and Q = number of rows (or columns) in a square matrix. The coefficient of intra-observer variation was established by the same observer (PMK) scoring 100 randomly selected fields 2 months after the first scoring. The inter-observer variation between 2 individuals (the author, PMK and Dr. R.G. Hegele) was determined from the simultaneous scoring of 100 random fields using a teaching microscope.

5.4 Photography.

Photomicrographs of selected lung sections were obtained on a Zeiss Photomicroscope III (Zeiss, West Germany). KG400 color films (Eastman Kodak Co, New Haven, CT) were used for photography of the sections and were sent to the film laboratory for processing and printing.

5.5 Statistical Analysis.

Statistical analyses were performed on anti-LMP1 mAb and anti-*bcl-2* mAb immunostaining data using SYSTAT® Version 5.1 software (Systat, Inc., Evanston, IL).

5.5.1 PCR Analysis.

The null hypothesis, that no difference existed in the mean number of copies

of EBV genome per cell among the 3 patient groups, was tested using the method of single factor analysis of variances (ANOVA). In the event of rejection of the null hypothesis, further analysis was carried out to investigate the nature of differences. The sequential rejective Bonferroni procedure was used to correct for multiple comparisons (172).

5.5.2 Analysis of EBV Density.

The presence of the EBV in a cell was interpreted by the staining of the viral protein LMP1 due to the EBV anti-LMP1 mAb; when a distinct signal was observed, it was considered positive and the absence of signal yielded negative data. The EBV status (positive versus negative) of the three groups was analyzed using contingency table analysis (χ^2 statistics) and a value of $p \leq 0.05$ was considered to be statistically significant. If the disease state of the 3 groups sampled was not independent of the EBV status, further analysis using Tukey-type testing was used (173). Of the cases that were EBV positive, the EBV density data were transformed by the logarithmic operation and analyzed by ANOVA and corrected for multiple comparisons using the sequential rejective Bonferroni procedure (172). Again, a value of $p \leq 0.05$ was considered to be statistically significant.

5.5.3 *bcl-2* Analysis.

The two histological features, cellularity and intensity, obtained using the human anti-*bcl-2* mAb were analyzed independently. ANOVA testing was applied to the null hypothesis that no difference existed in the mean cumulative cellularity score among the 3 sample populations. In the event of the rejection of the null hypothesis,

further analysis was carried out to determine the significant differences. To analyze the other histological feature, intensity, a similar null hypothesis using similar analytical procedures was set up to test the mean cumulative intensity score. A value of $p \leq 0.05$ was considered to be of statistical significance for both the above null hypotheses.

Figure 2. *bcl-2* scoring system.

Representative mid power photomicrographs of human lung tissue stained with human anti-*bcl-2* mAb (1 in 400 dilution) and graded according to the cellularity (x = 1,2,3) and the intensity (y = 0,1,2,3).

(Hematoxylin counterstained; bar represents 50 μm)

Panel a:Score = (1,0)

Panel b:Score = (2,0)

Panel c:Score = (3,0)

Panel d:Score = (1,1)

Panel e:Score = (2,1)

Panel f:Score = (3,1)

Panel g:Score = (1,2)

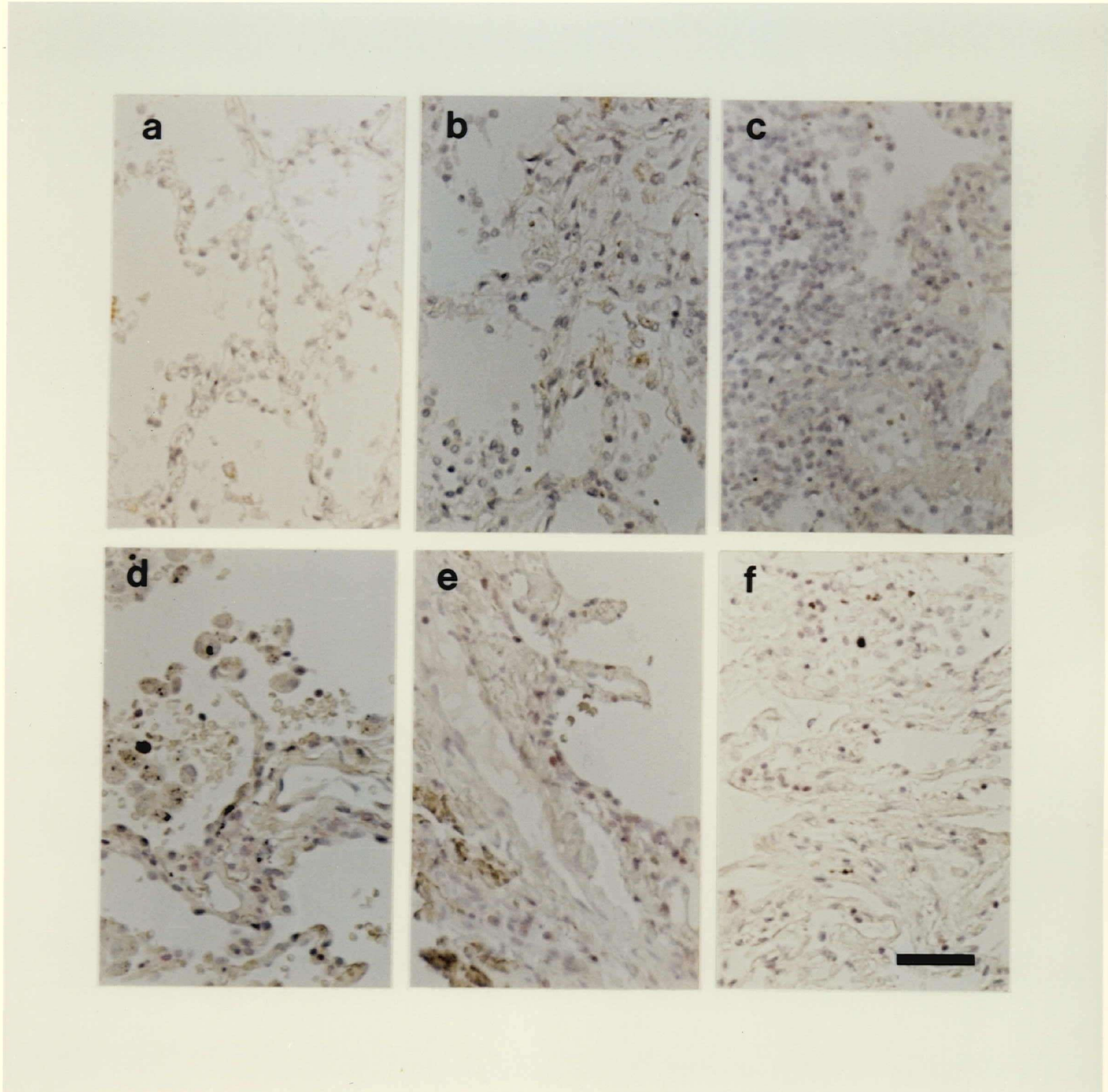
Panel h:Score = (2,2)

Panel i:Score = (3,2)

Panel j:Score = (1,3)

Panel k:Score = (2,3)

Panel l:Score = (3,3)


a
b
c
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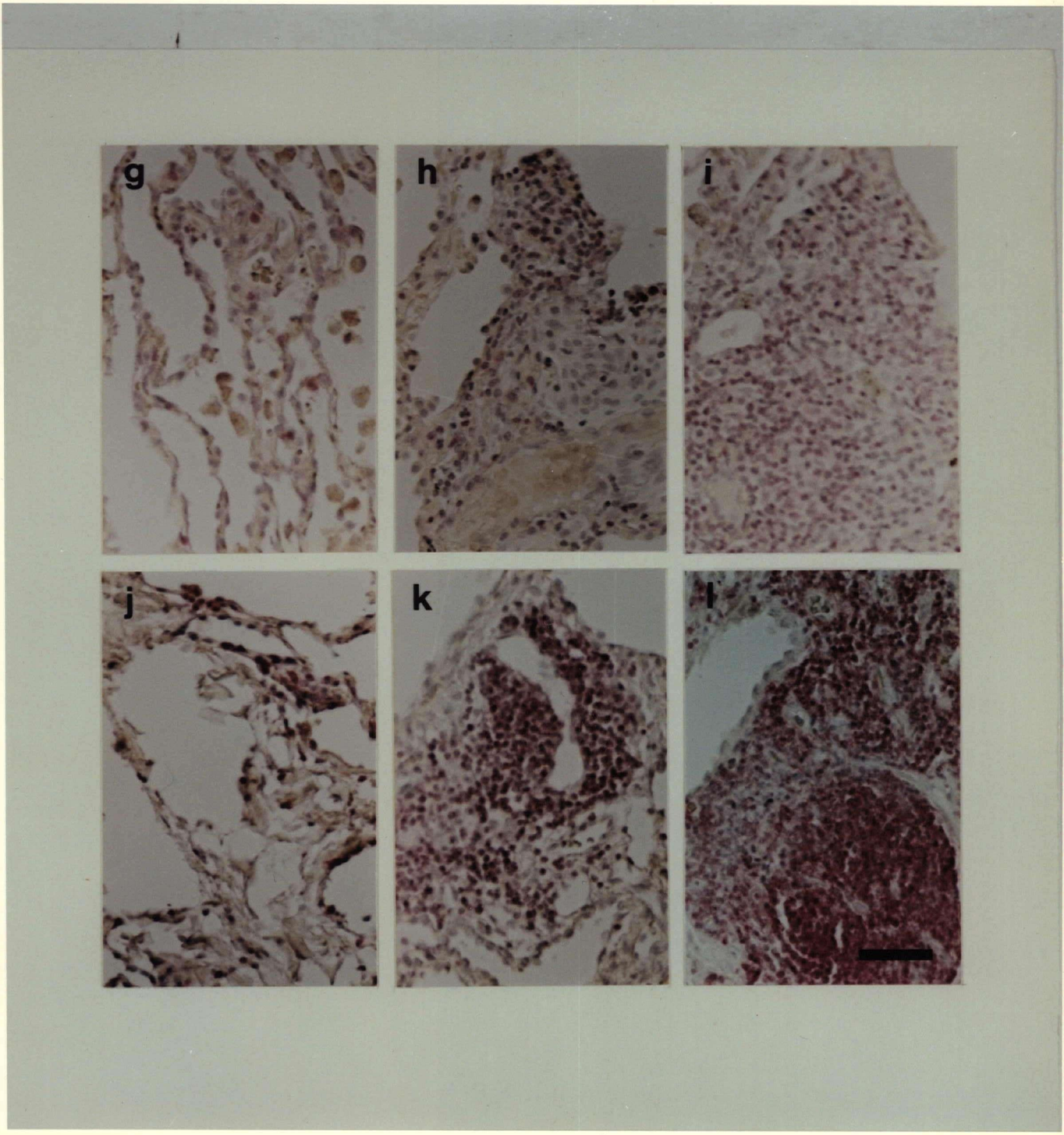
e
f


g
h
i
j
k
l

CHAPTER 6 RESULTS

All the necessary patient information and data collected during the course of this study and the relevant statistical analyses can be found in the Appendix. **6.1 Specific Aim 1: PCR Analysis.**

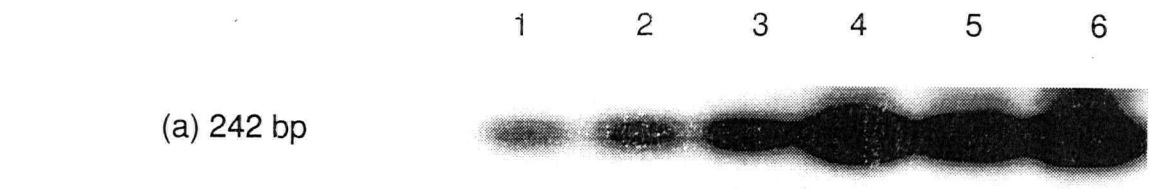
PCR amplification was calibrated using DNA extracted from Namalwa cells. Densitometric measurement of the autoradiographic band of PCR products probed by internal oligonucleotides was performed to obtain a linear relationship for calibration of both HLA DQα and EBV LMP1 PCR between 7 pg and 5000 pg of template DNA (Figure 3; Appendix II - Tables 7 and 8). Both PCR reactions reached a plateau when the DNA template was greater than the above range (data not presented).

Of the thirteen LIP cases, only one case yielded the 242 bp HLA DQα band. In the IPF group, four out of nine cases were HLA DQα positive, while in the AC group, all nine cases had the HLA DQα gene amplified.

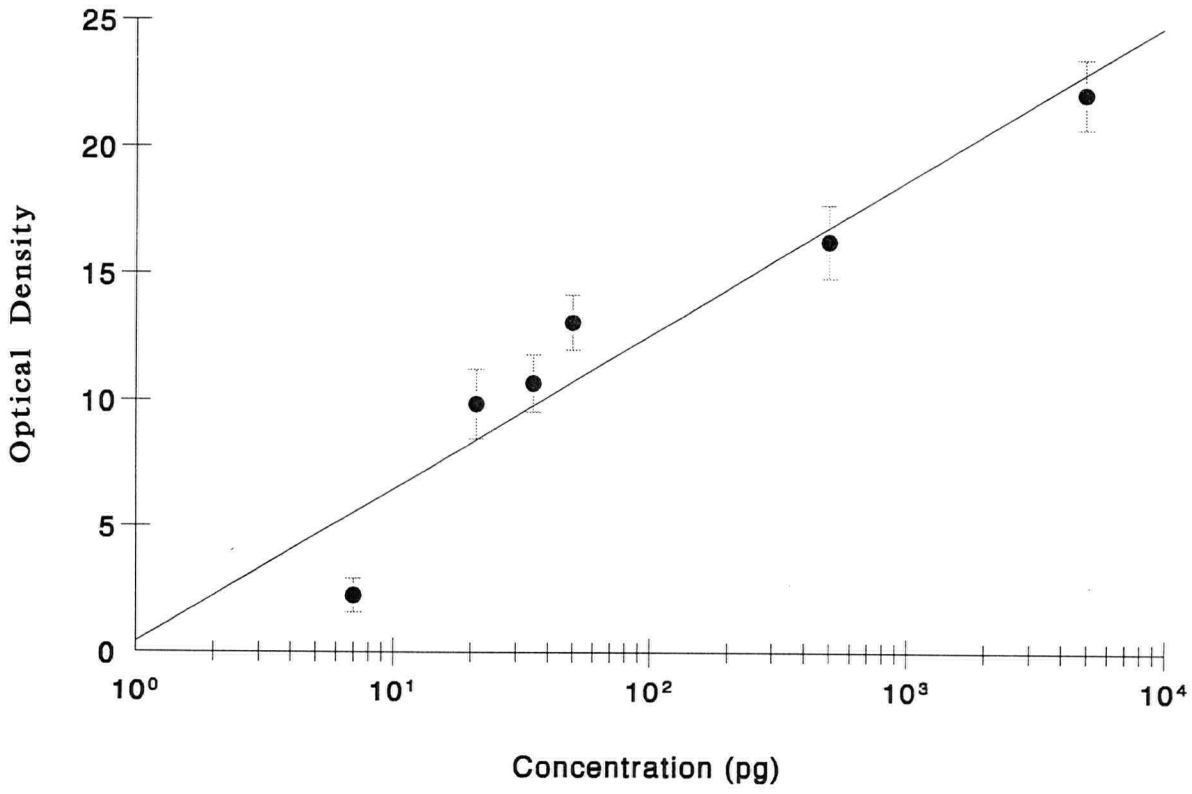
The one LIP case which was positive for HLA DQα was negative for the EBV PCR. Of the four HLA DQα positive cases from the IPF group, only one case was also EBV positive by PCR. Five out of nine AC cases were positive for EBV by PCR. Amplification of the EBV LMP1 gene was not carried out in those cases which were HLA DQα negative. Samples in which the DNA was not suitable for PCR consisted of the majority (12/13) of the LIP samples and 5/9 cases from the IPF group. PCR products of both HLA DQα and EBV LMP1 of selected cases are illustrated in Figure 4. PCR data are summarized in Appendix II, Table 9.

Figure 3. Calibration of PCR.

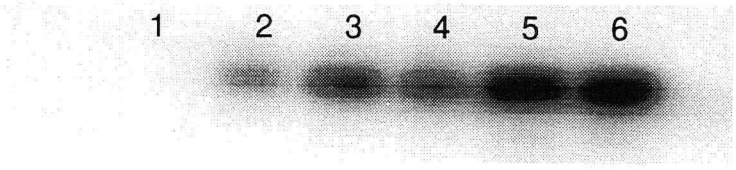
Standard curves correlating PCR product yield to template DNA concentration. PCR amplification of the (A) HLA DQα and (B) EBV LMP1 genes were calibrated using known amounts of DNA extracted from Namalwa cells. The intensity of the amplified products was determined by densitometric measurements of the band on the autoradiograph after Southern blot hybridization (a and b, respectively). A linear relationship was obtained for both amplification of the HLA DQα gene ($r^2 = 0.91$) and the EBV LMP1 gene ($r^2 = 0.95$). Mean $\pm$ SD of 3 replicates plotted (SD bars omitted if too small).


1
2
3
4
5
6
(a) 242 bp

(A)


25
20
15
10
5
0
Optical Density
10⁰
10¹
10²
10³
10⁴
Concentration (pg)

(b) 501 bp


1
2
3
4
5
6

(B)

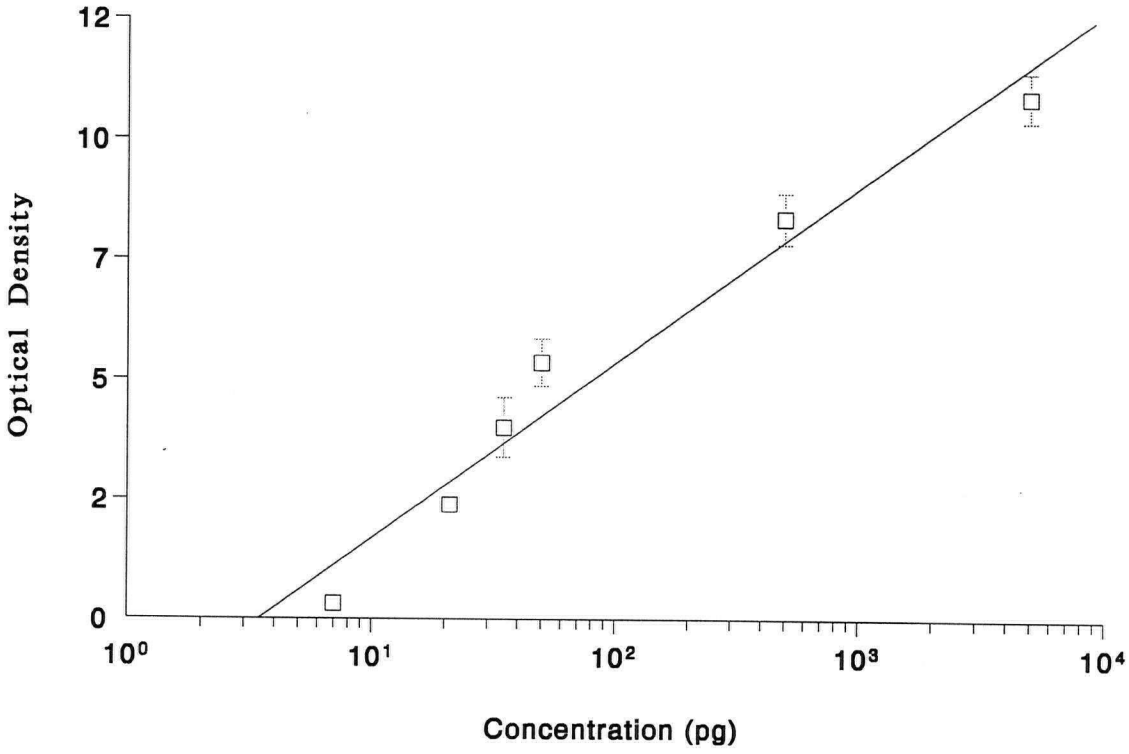
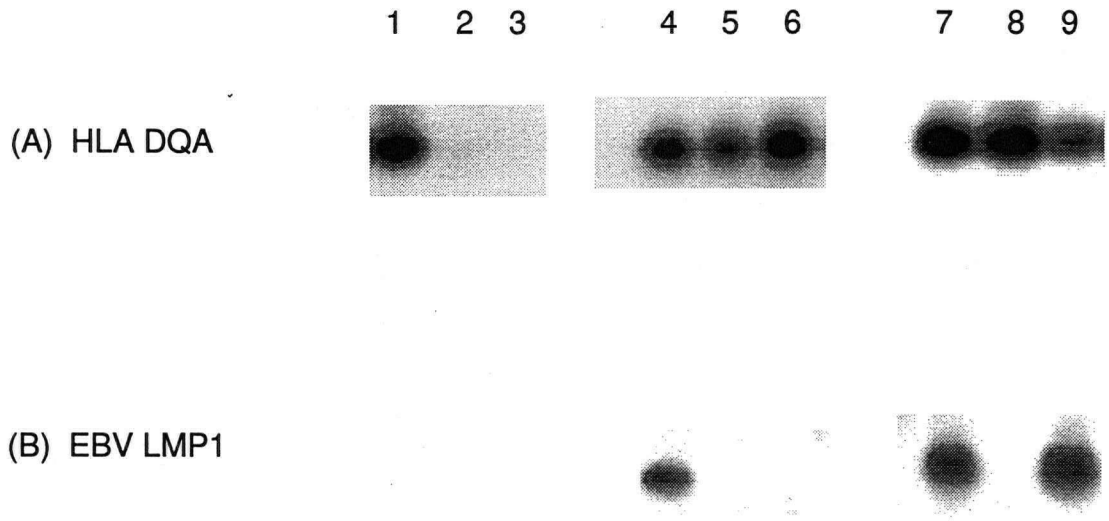

12
10
7
5
2
0
Optical Density
10⁰
10¹
10²
10³
10⁴
Concentration (pg)

Figure 4. Autoradiograph of PCR products.

Autoradiograph of the (A) HLA DQα product (242 bp) and the respective (B) EBV LMP1 product (501 bp) from selected LIP (lanes 1 to 3), IPF (lanes 4 to 6) and AC (lanes 7 to 9) patients. All samples presented were positive for amplification of the HLA DQα gene except for 2 LIP patients (lanes 2 and 3). The only HLA DQα positive LIP patient was negative for the EBV LMP1 PCR. DNA samples from one IPF (lane 4) and 2 AC (lanes 7 and 9) patients were EBV LMP1 PCR positive.


1 2 3
4 5 6
7 8 9
(A) HLA DQA
(B) EBV LMP1

6.2 Specific Aim 2: Immunostaining with EBV Anti-LMP1 mAb.

The Namalwa cell line tested positive for LMP1 by monoclonal Ab staining (Figure 5) while the BJAB cells were negative (data not presented). Diffuse cytoplasmic staining specific for the EBV LMP1 protein was observed in the Namalwa cells (Figure 5a) as well as in the positive tissue control, nasopharyngeal carcinoma (data not shown). LMP1 positive cells in the lung tissues from all 3 patient groups were of epithelial origin. Photomicrographs of a lung section from a patient with LIP stained with the anti-LMP1 mAb and non-specific IgG_1 are illustrated in Figure 5c and 5d, respectively. Diffuse LMP1 signals were localized in the apical region of epithelial cells lining the bronchioles. LMP1 signals were not observed in lymphoid cells in any of the patient samples.

The results of the immunostaining with EBV anti-LMP1 mAb are summarized in Table 3. Data pertaining to the calculation of EBV density was presented in Appendix II, Table 10. Immunostaining with the anti-LMP1 mAb was positive in ten of thirteen patients with LIP (77%) and three of nine cases in both control groups (33%). The incidence of LMP1-positive cells in lung tissue from LIP patients was significantly greater than those of the control groups (χ^2 statistics, $p \leq 0.05$). The mean EBV density of the positive cases in LIP patients was 14 times greater than those of the IPF patients and 20 times greater than those of the AC patients. ANOVA analysis of logarithmic-transformed positive EBV density were statistically significant (LIP versus IPF: $p \leq 0.01$, LIP versus AC: $p \leq 0.009$). There was no difference between the mean EBV density in lung tissues of IPF and AC patients.

Figure 5. Immunostaining with EBV anti-LMP1 mAb.

Photomicrographs of EBV LMP1 immunostaining of Namalwa cells (positive control, a and b) and lung tissue from a LIP patient (c and d). Panel a: Namalwa cells show strong membrane and cytoplasmic signals. Panel b: IgG_1 negative control. Panel c: EBV LMP1 protein was localized to the apical region of epithelial cells lining a bronchiole (B). Lymphoid cells within a nearby lymphoid aggregate (L) were negative. Panel d: IgG_1 negative control. (Counterstained with Mayer's hematoxylin; bar represents 50 μm)

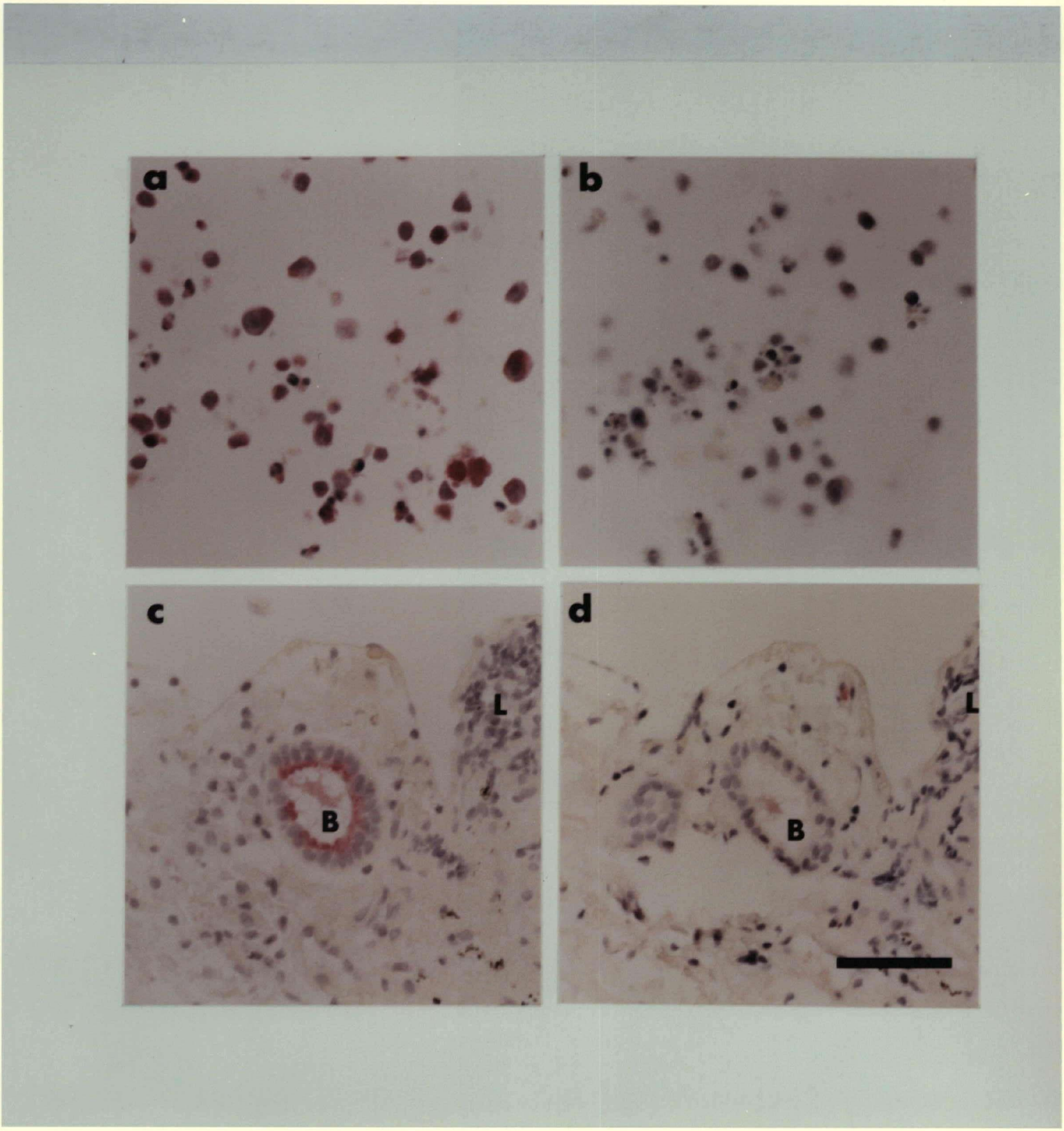

a
b
c
L
B
d
L
B

Table 3. Summary of Immunostaining with EBV anti-LMP1 monoclonal antibody.

		LIP	IPF	AC
No. of positive cases		10/13*	3/9	3/9
		(77%)	(33.3%)	(33.3%)
No. of positive blocks		20/26	5/20	3/18
		(77%)	(25%)	(17%)
EBV Density†				
	Mean	1.41 ± 1.14‡	0.10 ± 0.05	0.07 ± 0.04
	Range	0.13 - 3.72	0.03 - 0.16	0.04 - 0.12

* $p \leq 0.05$ LIP vs IPF and LIP vs AC, statistically significant.
† Based on LMP1 positive cases only.
‡ $p \leq 0.01$ LIP vs IPF and LIP vs AC, statistically significant.

6.3 Specific Aim 3: Immunostaining with human Anti-*bcl-2* mAb.

For all 3 patient groups, staining for the *bcl-2* protein was observed most commonly in cells of lymphoid origin. In all the lung sections, *bcl-2* signals were observed in several lymphocytes within the alveolar septa. In addition, lymphocytes within the lymphoid aggregates in lung tissue from patients with LIP were strongly positive for the anti-*bcl-2* mAb.

The distribution of the cumulative scores for cellularity and intensity of *bcl-2* staining in the lung tissue from the three patient groups are illustrated in Figure 6 while the corresponding data are presented in Appendix II, Table 11. Analysis by ANOVA showed that the mean cumulative cellularity score in LIP lung tissue was higher than that in IPF lung tissue ($p \leq 0.005$) and also higher than that of patients in

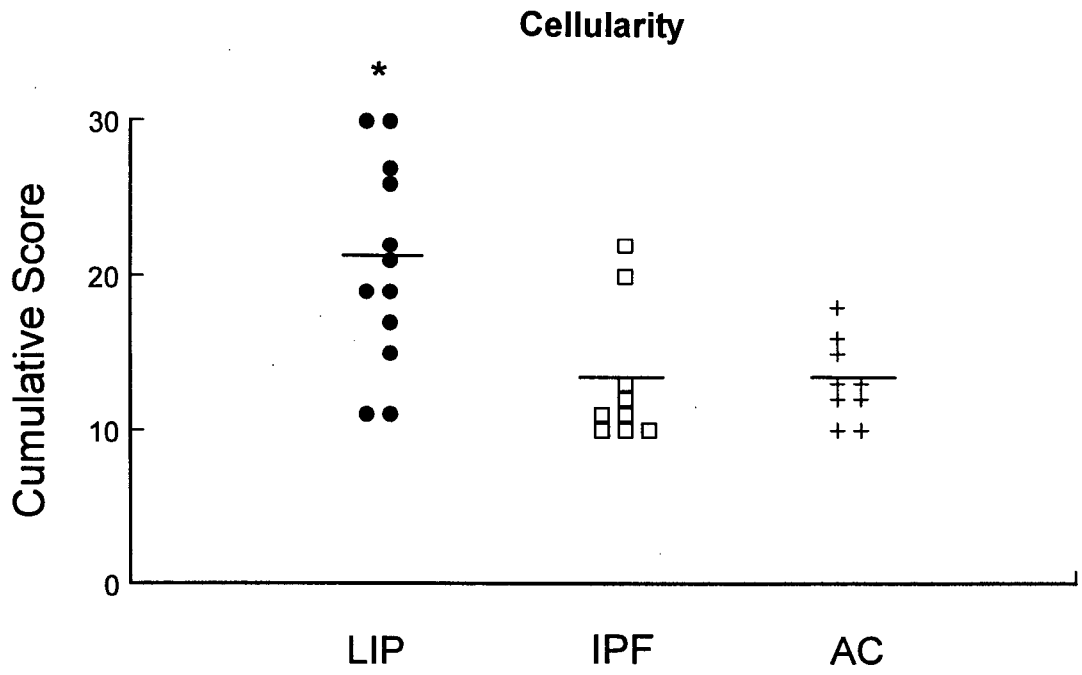
the AC group ($p \leq 0.005$). There was no significant difference in the mean cumulative cellularity scores of the IPF and AC control groups.

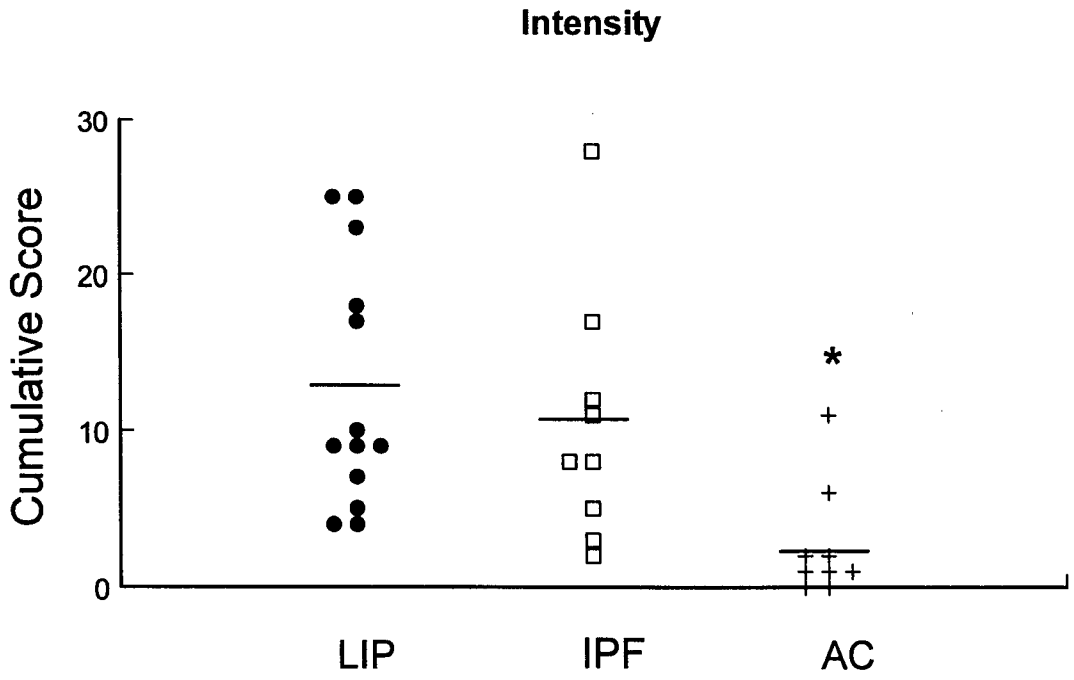
By the ANOVA analysis of the mean cumulative intensity scores, there was no difference between the LIP group and the IPF group ($p = 0.46$). However, in the comparison of the patients with interstitial lung disease with either LIP or IPF, to AC patients, the *bcl-2* cumulative intensity scores were significant (LIP versus AC, $p \leq 0.008$; and IPF versus AC, $p \leq 0.05$).

The reproducibility of the *bcl-2* scoring system was validated through the analysis of within- and between-observers values of R_2/R_{2max} for each histological feature. The R_2/R_{2max} value of intra-observer variation was calculated to be 0.98 for cellularity and 0.97 for intensity. The inter-observer R_2/R_{2max} value was 0.75 and 0.84 for cellularity and intensity, respectively.

Figure 6. Analysis of *bcl-2* staining.

Paraffin sections of lung samples from LIP samples (●, n=13), IPF (□, n=9) and AC (+, n=9) patients were stained with anti-*bcl-2* mAb and scored for Cellularity (top panel, * $p \leq 0.005$) and Intensity (bottom panel, * $p \leq 0.05$) based on the semi-quantitative scale described in Materials and Methods. Each symbol represents the cumulative score from the 10 random fields examined for each individual. The mean of the cumulative scores of each patient group is indicated by a horizontal bar.


Cellularity
Cumulative Score
30
20
10
0
*
LIP
IPF
AC


Intensity
Cumulative Score
30
20
10
0
*
LIP
IPF
AC

CHAPTER 7 DISCUSSION

The final chapter of this thesis compares the results of the present study to those of previous studies, focusing mainly on the consistency and/or contradictions between studies and the relevant implications. Because of technical difficulties, PCR has not been useful in obtaining informative data for determination of the prevalence of EBV and estimation of the number of copies of EBV genomes per cell in lung tissue of patients with LIP compared to controls. The results based on immunohistochemistry showed that EBV LMP1 was more prevalent with an increased EBV density in LIP patients ($p \leq 0.01$). Furthermore, LIP and IPF lung tissue had increased expression of *bcl-2* in comparison to AC ($p \leq 0.05$). These results support the hypothesis that EBV plays a role in the pathogenesis of LIP via expression of the viral latent protein, LMP1, and that over-expression of the cellular *bcl-2* gene may be involved.

7.1 Specific Aim 1: Polymerase Chain Reaction.

Amplification of the single-copy human gene, HLA DQα, was successful in only one out of thirteen DNA samples extracted from patients with LIP and four out of nine from those in the IPF group. The one LIP case that was HLA DQα positive was EBV LMP1 negative by PCR. This particular case was a recent addition to the LIP series in which most of the cases were collected over twenty years ago. Of the four IPF cases that were positive for HLA DQα PCR, only one case tested positive in the EBV

LMP1 PCR. Amplification of another housekeeping gene such as the β-globin gene[5] on the DNA samples which were negative by HLA PCR were likewise negative. Failure to amplify essential housekeeping genes may be attributed to the integrity of the preserved DNA, which may in turn be affected by the age of the formalin-fixed lung tissue[6]. Further PCR experiments[7] were carried out on the HLA DQα negative samples "spiked" with a fixed amount of extraneous human placenta DNA (174). The results showed that an increasing amount of DNA from patients with LIP/IPF caused a corresponding decrease in the amplification of the extraneous DNA. This finding indicated the likelihood of the presence of PCR inhibitor(s) which may interfere with the activity of the Taq polymerase. To evaluate this possibility, Centricon filters[8]

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[5]The β-globin PCR was carried out in a total volume of 50 μl containing 25 pmol primers (GH20 and PC04 (Perkin Elmer, gift from Dr. M. Daya)), 1.25 mM of dATP, dCTP, dGTP and dTTP each, 10 mM Tris HCl (pH 8.4), 50 mM KCl, 2.5 mM $MgCl_2$ and 1.25 units of Taq DNA polymerase. The cycling conditions were 94°C 1 min, 65°C 2 min and 72°C 1 min for 40 cycles using the PCR System 9600. These conditions were optimized by Dr. Daya and coworkers.

[6]A similar situation had been observed by Dr. K. Kuwano (Pulmonary Research Laboratory, UBC).

[7]DNA samples extracted from patients with LIP/IPF which were HLA DQα PCR negative were used as a source of genomic DNA. Increasing amounts of genomic DNA (1, 5, 15 and 30 μl) were amplified in the presence/absence of extraneous DNA (50 pg human placenta DNA) using HLA DQα primers.

[8]DNA samples from LIP/IPF patients were extracted as described in Section 5.2.3. and dissolved in 100 μl distilled water. Samples were then additionally extracted once with equal volume of phenol (equilibrated with 100 mM Tris HCl (pH 8.0), 0.1% hydroxyquinoline), once with 1:1 mixture of phenol and chloroform and once with chloroform. The supernatant was centrifuged through Centricon-30 membrane (Amicon, UK; donated by Dr. B.M. Walker) by using a Beckman JA-14 rotor (angle 25°) at 5000 x g for 30 min. The final retentate was used for amplification of HLA DQα gene.

were employed in an attempt to remove any PCR inhibitors that may be present (174). Amplification of the Centricon-filtered DNA with HLA DQα primers remained negative. Consequently, it was not possible to draw a conclusion about the EBV status in these samples by the PCR technique.

Amplification of the HLA DQα gene was successful in all nine AC cases of which five were also EBV LMP1 positive. Although we were unable to compare these results with that of the interstitial lung disease samples, the prevalence of EBV in lung tissue of AC controls agree with the PCR data published by Cheung *et al* (175) in which five out of nine normal subjects were EBV positive. This apparently high rate of detection of EBV DNA in "normal" subjects is a plausible finding considering the high proportion (>95%) of EBV-seropositive individuals in the general population (176).

7.2 Specific Aim 2: Immunostaining with EBV anti-LMP1 mAb.

In this thesis, immunohistochemistry localized the expression of the EBV latency gene, LMP1, to the respiratory epithelium of lung tissues in LIP patients and the control IPF and AC patients. The EBV prevalence was found to be greater in the LIP group than in the controls. When the scoring system used to define the EBV density was applied to the lung tissues that were positive for anti-LMP1 mAb, the EBV density in LIP patients was 14 to 20 times higher than controls.

The overall result that EBV is more prevalent in LIP patients than controls is consistent with the finding of Barberá *et al* (24). These investigators demonstrated EBV DNA in nine out of fourteen cases of lung tissue from LIP patients compared to

two out of ten IPF patients by *in situ* hybridization (24). However, an apparent discrepancy is observed in the localization of signals in different cell types when using different techniques. By *in situ* hybridization, Barberá *et al* (24) identified EBV DNA harboring cells to be of mainly lymphoid origin along the alveolar septa and less frequently within the lymphoid aggregates. In contrast, the present study, localized EBV-encoded LMP1 to epithelial cells lining respiratory bronchioles. The different distribution of EBV genome and protein might be one way in which the EBV evades the host immune surveillance, since determinants from LMP1 may be recognized by CD8 T lymphocytes in the context of specific class I MHC molecules (177). Down-regulation of LMP1 in B lymphocytes would therefore prolong survival of EBV-infected B cells and facilitate tumor development (54).

The finding of LMP1 signals in epithelial cells of respiratory bronchioles in both control groups by immunohistochemistry, as discussed previously (Section 7.1), is a reflection of the high prevalence of EBV-seropositive individuals. However, the scoring system employed in this thesis revealed a higher EBV density in LIP lungs compared to controls. As the IPF controls had a similar EBV prevalence and EBV density as the AC controls, this suggests that IPF, unlike LIP, is not associated with EBV infection.

Immunohistochemical labelling with anti-LMP1 mAb is a simple and useful technique that allowed the detection of the viral latent membrane protein LMP1 in paraffin-embedded lung tissues. In addition, the scoring system designed in this thesis estimated the EBV density. Two major advantages immunohistochemistry has

over PCR is that the former not only allows the examination of the preserved tissue architecture but also confirms the active expression of the gene investigated within specific cells. Immunohistochemistry may therefore be used as an alternative method of investigation in situations in which the samples are not suitable for PCR.

7.3 Specific Aim 3: Immunostaining with human anti-*bcl-2*.

The *bcl-2* immunohistochemical data indicate that lung tissue from LIP patients had the greatest number of lymphoid cells displaying strong *bcl-2* signals. In comparison, there were significantly fewer *bcl-2* positive lymphoid cells in the IPF controls with similar intensity and the AC group had few weakly positive *bcl-2* cells of lymphoid origin. These results suggest that constitutive expression of *bcl-2* may contribute to lymphomagenesis in LIP and that dysregulation of *bcl-2* expression may play a role in the pathogenesis of LIP.

High level expression of *bcl-2* is frequently observed in a wide spectrum of LPD (26,27,141,146,147,178) and is anticipated in LIP. In these previous reports *bcl-2* expression was analyzed by mRNA studies and was measured by semi-quantitative methods. In the present study, only formalin-fixed paraffin-embedded lung tissues were available, hence detection by immunohistochemistry was more appropriate. However, analysis of overall *bcl-2* expression can only be determined by semi-quantitative methods such as one used in this thesis. In the scoring system described in Section 5.3.6, the two histological features, cellularity and intensity, are both essential for determination of the level of *bcl-2* expression. Therefore, a significant increase in either cellularity or intensity would indicate in significant increase in the

level of *bcl-2* expression.

The association between *bcl-2* and LIP has not been previously reported. As has been proposed for a large proportion of hematopoietic neoplasms, it is plausible that elevated *bcl-2* expression confers survival advantage and protects against apoptosis of *bcl-2* positive neoplastic cells. Extended cell survival could provide an increased opportunity for acquiring secondary genetic defects in growth and proliferation genes and/or tumor suppressor genes (124). In addition, a prolonged B cell life span has been implicated in the pathogenesis of lymphoid neoplasia in studies using transgenic mice (179). Similarly, cells which display intense *bcl-2* staining in the lung tissue of LIP patients may be protected from apoptosis. The accumulation of persistent lymphoid elements within the lymphoid aggregates of LIP lung tissue may be related to the survival advantage conferred by *bcl-2* and these mechanisms could be involved in the progression of LIP to low- or mid-grade non-Hodgkin's lymphoma.

In the IPF series, a small proportion of cells with intense *bcl-2* staining was observed. The accumulation of a considerably smaller number of long-lived cells may have a lesser effect in the disruption of normal tissue homeostasis and suggests that accumulation of long-lived lymphoid elements leading to malignant lymphoma is unlikely. However, the significance of the presence of a minority of cells with high levels of *bcl-2* protein remains unclear.

Concerning the clinical implications of the current findings, over-expression of *bcl-2* has been reported to confer a worse prognosis in non-Hodgkin's lymphoma (180), acute myelogenous leukemia (181) and prostate carcinoma (182). Whether the

same holds true for the prognosis of LIP could not be determined since LIP is not a major cause of death in these patients. The association of LIP with aberrant expression of *bcl-2*, as presented in this thesis, suggests that the routine management of LIP involving steroid treatment and/or chemotherapy (3) be re-evaluated since cell death induced by these agents is inhibited by *bcl-2* expression (28).

7.4 Comments and Conclusion.

The experiments performed in this thesis were designed to explain the possible role of latent EBV infection in the etio-pathogenesis of LIP mediated through increased expression of *bcl-2*, an approach other investigators have used for a variety of other neoplasms (183-186). However, in contrast to the *in vitro* demonstration of upregulation of *bcl-2* by the EBV LMP1, none of these studies has conclusively demonstrated a direct relationship between LMP1 and *bcl-2* expression. Although the results obtained in this thesis demonstrated the higher prevalence of the expression of EBV LMP1 gene as well as the over-expression of the cellular *bcl-2* proto-oncogene in lung tissues of LIP patients, the immunohistochemical staining localized the EBV LMP1 signals to epithelial cells of the respiratory bronchioles while *bcl-2* signals were localized to lymphoid cells. Since the immuno-signals of *bcl-2* and LMP1 are generally manifested in different cell types in the human lung tissue, it is therefore unlikely that the expression of these two proteins are directly linked.

A general and simplistic view of LPD could be described by the equation (28):

Rate of cell accumulation

= Rate of cell proliferation - Rate of cell death

Excess cells accumulate either by increasing the rate at which cells divide or by decreasing the frequency with which cells die. In LIP, the higher prevalence of EBV associated with this disease suggests the likelihood of an increase in cell proliferation due to immortalization of B cells by the virus. At the same time, high level expression of *bcl-2* inhibits programmed cell death. Therefore, lymphoproliferation in LIP is compounded by both the role of EBV and the effect of high level *bcl-2* expression.

In conclusion, the EBV may play a role in the pathogenesis of LIP mediated through the expression of LMP1 and constitutive expression of *bcl-2* could contribute to the tumorigenesis of LIP.

REFERENCES

1. Liebow, A. A. and C. B. Carrington. 1973. Diffuse pulmonary lymphoreticular infiltrations associated with dysproteinemia. *Med Clin North Am* 57(3):809-843.

2. Schwarz, M. I. 1992. *Lymphoplasmocytic interstitial pneumonias*. In: Interstitial lung disease. Schwarz, M. I. and T. E. King Jr., eds. Mosby Year Book, 405-412.

3. Pitt, J. 1991. Lymphocytic interstitial pneumonia. *Childhood AIDS* 38(1):89-95.

4. Faguet, G. B., H. H. Webb, J. F. Agee, W. B. Ricks and A. H. Sharbaugh. 1978. Immunologically diagnosed malignancy in Sjogren's pseudolymphoma. *Am J Med* 65:424-429.

5. Bonner, H. and R. S. Ennis. 1973. Lymphoid infiltration and amyloidosis of lung in Sjogren's syndrome. *Arch Pathol* 95:42-44.

6. Joshi, V. V., J. M. Oleske and A. B. Minnefor. 1985. Pathologic pulmonary findings in children with the acquired immunodeficiency syndrome: a study of ten cases. *Hum Pathol* 16:241-246.

7. Katz, B. Z., A. B. Berkman and E. D. Shapiro. 1992. Serologic evidence of active Epstein-Barr virus infection in Epstein-Barr virus-associated lymphoproliferative disorders of children with acquired immunodeficiency syndrome. *J Pediatr* 120:228-232.

8. Kramer, M. J., M. J. Saldana, M. Ramos and A. E. Pitchenik. 1992. High titers of Epstein-Barr virus antibodies in adult patients with lymphocytic interstitial pneumonitis associated with AIDS. *Respir Med* 86:49-52.

9. Solal-Celigny, P., L. J. Couderc, D. Herman, P. Herve, L. Schaffar-Deshayes, F. Brun-Vezinet, G. Tricot and J. P. Clauvel. 1985. Lymphoid interstitial pneumonitis in acquired immunodeficiency syndrome-related complex. *Am Rev Respir Dis* 131:956-960.

10. Anonymous, A. 1990. *Primary lymphoid lung lesions*. In Surgical pathology of non-neoplastic lung disease. Katzenstein, A. L. A. and F. B. A. Askin, eds. WB Saunders Company, 290-320.

11. Centers for Disease Control: 1987. Revision of the case definition of acquired immunodeficiency syndrome. Council of State and Territorial Epidemiologists. *MMWR* 36:1S-15S.

12. Saldana, M. J. and J. M. Mones. 1992. Lymphoid interstitial pneumonia in HIV

infected individuals. *Prog Surg Pathol* 12:181-215.

13. Oleske, J., A. Minnefor, R. Jr. Cooper, K. Thomas, A. dela Cruz, H. Ahdieh, I. Guerrero, V. V. Joshi and F. Desposito. 1983. Immune deficiency syndrome in children. *JAMA* 249:2346-2349.

14. Goldman, H. S., M. N. Ziprkowski and M. Charytan. 1985. Lymphocytic interstitial pneumonitis in children with AIDS: a perfect radiographic-pathologic correlation. *AJR* 145:868A.

15. Anderson, V. M. and H. Lee. 1988. Lymphocytic interstitial pneumonitis in pediatric AIDS. *Pediatric Pathol* 8:417-421.

16. Fox, R. I., G. Pearson and J. H. Vaughan. 1986. Detection of Epstein-Barr virus-associated antigens and DNA in salivary gland biopsies from patients with Sjogren's syndrome. *J Immunol* 137:3162-3168.

17. Saito, I., B. Servenius, T. Compton and R. I. Fox. 1989. Detection of Epstein-Barr virus DNA by polymerase chain reaction in blood and tissue biopsies from patients with Sjogren's syndrome. *J Exp Med* 169:2191-2198.

18. Randhawa, P. S., S. A. Yousem, I. L. Paradis, J. A. Dauber, B. P. Griffith and J. Locker. 1989. The clinical spectrum, pathology, and clonal analysis of Epstein-Barr virus-associated lymphoproliferative disorders in heart-lung transplant recipients. *Am J Clin Pathol* 92:177-185.

19. Hanto, D. W., G. Frizizera, K. J. Gajl-Peczalska and R. L. Simmons. 1985. Epstein-Barr virus, immunodeficiency and B cell lymphoproliferation. *Transplantation* 39:461-472.

20. Sullivan, J. L. 1988. Epstein-Barr virus and lymphoproliferative disorders. *Semin Hematol* 25:269-279.

21. Katz, B. Z. and U. Saini. 1992. Presence of the diffuse early antigen of Epstein-Barr virus in lymphomas and lymphoproliferative disorders. *Am J Pathol* 140:1247-1254.

22. Randhawa, P. S., J. Demetris and M. A. Nalesnik. 1994. EBER gene expression in Epstein-Barr virus-associated hematopoietic neoplasms. *Leukemia and Lymphoma* 13:387-392.

23. Andiman, W. A., K. Martin, A. Rubinstein, S. Pahwa, R. Eastman, B. Z. Katz, J. Pitt and G. Miller. 1985. Opportunistic lymphoproliferations associated with Epstein-Barr viral DNA in infants and children with AIDS. *Lancet* 2:1390-1393.

24. Barbera, J. A., S. Hayashi, R. G. Hegele and J. C. Hogg. 1992. Detection of Epstein-Barr virus in lymphocytic interstitial pneumonia by *in situ* hybridization. *Am Rev Respir Dis* 145:940-946.

25. Pezzella, F., A. G. D. Tse, J. L. Cordell, K. A. F. Pulford, K. C. Gatter and D. Y. Mason. 1990. Expression of the *bcl-2* oncogene protein is not specific for the 14;18 chromosomal translocation. *Am J Pathol* 137:225-232.

26. Zutter, M., D. Hockenbery, G. A. Silverman and S. J. Korsmeyer. 1991. Immunolocalization of the *bcl-2* protein within hematopoietic neoplasms. *Blood* 78(4):1062-1068.

27. Mariano, M. T., L. Moretti, A. Donelli, M. Grantini, G. Montagnani, A. U. DiPrisco, G. Torelli, U. Torelli and F. Narni. 1992. *Bcl-2* gene expression in hematopoietic cell differentiation. *Blood* 80(3):768-775.

28. Reed, J. C. 1994. Mini-review: Cellular mechanisms of disease series. *Bcl-2* and the regulation of programmed cell death. *J Cell Biol* 124:1-6.

29. Young, L., C. Alfieri, K. Hennessy, H. Evans, C. O'Hara, K. C. Anderson, J. Ritz, R. S. Shapiro, A. Rickinson, E. Kieff and J. I. Cohen. 1989. Expression of Epstein-Barr virus transformation-associated genes in tissues of patients with EBV lymphoproliferative disease. *N Engl J Med* 321:1080-1085.

30. Subar, M., A. Neri, G. Inghirami, D. M. Knowles and R. Dalla-Favera. 1988. Frequent c-myc oncogene activation and infrequent presence of Epstein-Barr virus genome in AIDS-associated lymphoma. *Blood* 72:667-671.

31. Jason, J., J. Stehr-Green and R. Holman. 1988. Human immunodeficiency virus infection in hemophilic children. *Pediatrics* 82(4):565-570.

32. Pizzo, P., J. Eddy and J. Faloon. 1988. Acquired immune deficiency syndrome in children. *Am J Med* 85(Suppl 2A):195-202.

33. Burkitt, D. 1958. A sarcoma involving the jaws in African children. *Br J Surg* 46:218-223.

34. Burkitt, D. 1962. Determining the climatic limitations of a children's cancer common in Africa. *Br Med J* 2:1019-1023.

35. Alfieri, C., F. Ghibu and J. H. Joncas. 1984. Lytic, non-transforming Epstein-Barr virus (EBV) from a patient with chronic active EBV infection. *Can Med Assoc J* 131:1249-1252.

36. Pulvertaft, R. J. V. 1964. Cytology of Burkitt's tumour (African lymphoma). *Lancet* 1:238-240.

37. Kieff, E. and D. Liebowitz. 1990. *Epstein-Barr virus and its replication*. In: Virology. Fields, B. N. and D. M. Knipe *et al.*, eds. Raven Press, New York. 1889-1920.

38. Galloway, J. 1989. Chance and felicity. *Nature* 338:463-464.

39. McClain, K. L., C. T. Leach, H. B. Jenson, V. V. Joshi, B. H. Pollock, R. T. Parmley, F. J. DiCarlo, E. G. Chadwick and S. B. Murphy. 1995. Association of Epstein-Barr virus with leiomyosarcomas in young people with AIDS. *N Engl J Med* 332:12-18.

40. Lee, E. S., J. Locker, M. Nalesnik, J. Reyes, R. Jaffe, M. Alashari, B. Nour, A. Tzakis and P. S. Dickman. 1995. The association of Epstein-Barr virus with smooth-muscle tumors occuring after organ transplantation. *N Engl J Med* 332:19-25.

41. Dolyniuk, M., R. Pritchett and E. D. Kieff. 1976. Proteins of Epstein-Barr virus. I. Analysis of the polypeptides of purified enveloped Epstein-Barr virus. *J Virol* 17:935-949.

42. Dolyniuk, M., E. Wolff and E. D. Kieff. 1976. Proteins of Epstein-Barr virus. II. Electrophoretic analysis of the polypeptide of the nucleocapsid and the glocosamine- and polysaccharide-containing components of enveloped virus. *J Virol* 18:289-297.

43. Thorley-Lawson, D. A. and C. M. Edson. 1979. Polypeptides of the Epstein-Barr virus membrane antigen complex. *J Virol* 32:458-467.

44. Dambaugh, T., C. Beisel, M. Hummel, W. King, S. Fennewald, A. Cheung, M. Heller, N. Raab-Traub and E. Kieff. 1980. EBV DNA. VII. Molecular cloning and detailed mapping of EBV (B95-8) DNA. *Proc Natl Acad Sci (USA)* 77:2999-3003.

45. Baer, R., A. T. Bankier, M. D. Biggin, P. L. Deininger, P. J. Farrell, T. J. Gibson, G. Hatfull, G. S. Hudson, S. C. Satchwell, C. Seguin, P. S. Tuffnell and B. G. Barrell. 1984. DNA sequence and expression of the B95-8 Epstein-Barr virus genome. *Nature* 310:207-211.

46. Hatfull, G., A. T. Bankier, B. G. Barrell and P. J. Farrell. 1988. Sequence analysis of Raji Epstein-Barr virus DNA. *Virology* 164:334-340.

47. Cohen, J. I. 1995. Molecular biology of Epstein-Barr virus and its mechanism of

B-cell transformation. In: Straus SE, moderator. Epstein-Barr virus infections: biology, pathogenesis and management. *Annals of Internal Medicine* 118:45-58.

48. Farrell, P. J., A. Bankier, C. Seguin, P. Deininger and B. G. Barrell. 1983. Latent and lytic cycle promoters of the Epstein-Barr virus. *EMBO J* 2:1331-1338.

49. Middleton, T., T. A. Gahn, J. M. Martin and B. Sugden. 1991. Immortalizing genes of Epstein-Barr virus. *Advances in Virus Research* 40:19-55.

50. Liebowitz, D. and E. Kieff. 1993. *Epstein-Barr Virus*. In The Human Herpesvirus. Roizman, B., R. J. Whitley and C. Lopez, eds. Raven Press, New York. 107-172.

51. Sixbey, J. W., E. H. Vesterinen, J. G. Nedrud, N. Raab-Traub, L. A. Walton and J. S. Pagano. 1984. Epstein-Barr virus replication in oropharyngeal epithelial cells. *N Engl J Med* 310:1225-1230.

52. Klein, G. 1994. Epstein-Barr virus strategy in normal and neoplastic B cells. *Cell* 77:791-793.

53. Gerber, P., F. Nkrumah, R. Pritchett and E. D. Kieff. 1976. Comparative studies of Epstein-Barr virus strains from Ghana and the United States. *Int J Cancer* 17:71-81.

54. Straus, S. E., J. I. Cohen, G. Tosato and J. Meier. 1993. Epstein-Barr virus infections: biology, pathogenesis, and management. *Annals Internal Med* 118:45-58.

55. Bauer, G., P. Hofler and H. zur Hausen. 1982. Epstein-Barr virus induction by a serum factor. I. Induction and cooperation with additional inducers. *Virology* 121:184-194.

56. Takada, K. 1984. Cross-linking of cell surface immunoglobulin induces Epstein-Barr virus in Burkitt's lymphoma lines. *Int J Cancer* 33:27-32.

57. Datta, A. K., R. J. Feighny and J. S. Pagano. 1980. Induction of Epstein-Barr virus-associated DNA polymerase by 12-O-tetradecanoylphorbol-13-acetate. *J Biol Chem* 255:5120-5125.

58. Hutt-Fletcher, L. M., E. Fowler, J. D. Lambris, R. J. Feingey, J. G. Simmons and G. D. Ross. 1983. Studies of the Epstein-Barr virus receptor found on Raji cells. II. A comparison of lymphocyte binding sites for Epstein-Barr and C3d. *J Immunol* 130:1309-1312.

59. Weis, J. J., L. E. Toothaker, J. A. Smith, J. H. Weis and D. T. Fearon. 1988. Structure of the human B lymphocyte receptor for C3d and the Epstein-Barr virus and

relatedness to other members of the family of C3/C4 binding proteins. *J Exp Med* 167:1047-1066.

60. Tanner, J., J. Weis, D. Fearon, Y. Whang and E. Kieff. 1987. Epstein-Barr virus gp350/220 binding to the B lymphocyte C3d receptor mediates adsorption, capping, and endocytosis. *Cell* 50:203-213.

61. Molina, H., C. Brenner, S. Jacobi, J. Gorka, J. C. Carel, T. Kinoshita and V. M. Holers. 1991. Analysis of Epstein-Barr virus-binding sites on complement receptor 2 (CR2/CD21) using human-mouse chimeras and peptides. At least two distinct sites are necessary for ligand-receptor interaction. *J Biol Chem* 266:12173-21279.

62. Moore, M. D., M. J. Cannon, A. Sewall, M. Finlayson, M. Okimoto and G. R. Nemerow. 1991. Inhibition of Epstein-Barr virus infection *in vitro* and *in vivo* by soluble CR2 (CD21) containing two short consensus repeats. *J Virol* 65:3559-3565.

63. Dales, S. and Y. Chardonet. 1973. Early events in the interaction of adenoviruses with Hela cells. IV. Association with microtubules and the nuclear pore complex during vectorial movement of the inoculum. *Virology* 56:465-483.

64. Ahearn, J. M., S. D. Hayward, J. C. Hickery and D. T. Fearon. 1988. Epstein-Barr virus (EBV) infection of murine L cells expressing recombinant human EBV/C3d receptor. *Proc Natl Acad Sci (USA)* 85:9307-9311.

65. Bankier, A. T., P. L. Deininger, P. J. Farrell and B. G. Barrell. 1983. DNA sequence analysis of EcoR1 Dhet fragment of B95-8 Epstein-Barr virus containing the terminal repeat sequences. *Mol Biol Med* 1:425-446.

66. Wolf, H., C. Bogedain and F. Schwarzmann. 1993. Epstein-Barr virus and its interaction with the host. *Intervirol* 35:26-39.

67. Marschall, M., F. Schwarzmann, U. Leser, B. Oker, P. Alliger, H. Mairhofer and H. Wolf. 1991. The BI'LF4 transactivator of Epstein-Barr virus is modulated by type and differentiation of the host cell. *Virology* 181:172-179.

68. Adams, A. and T. Lindahl. 1975. Epstein-Barr virus genomes with properties of circular DNA molecules in carrier cells. *Proc Natl Acad Sci (USA)* 72:1477-1481.

69. Lindahl, T., A. Adams, G. Bjursell, G. W. Bornkamm, C. Kaschka-Dierich and U. Jehn. 1976. Covalently closed circular duplex DNA of Epstein-Barr virus in a human lymphoid cell line. *J Mol Biol* 102:511-530.

70. Lindahl, T., G. Klein, B. M. Reedman, B. Johansson and S. Sing. 1974. Relationship between Epstein-Barr virus DNA and the EBV-determined nuclear antigen

(EBNA) in Burkitt's lymphoma biopsies and other lymphoproliferative malignancies. *Int J Cancer* 13:764-772.

71. Alfieri, C., M. Birkenbach and E. Kieff. 1991. Early events in Epstein-Barr virus infection of human B-lymphocytes. *Virology* 181:595-608.

72. Hurley, E. A. and D. Thorley-Lawson. 1989. B cell activation and the establishment of EBV latency. *J Exp Med* 168:2059-2075.

73. Miller, G. 1990. *Epstein-Barr virus: Biology, pathogenesis, and medical aspects*. In Virology. Fields, B. N. and D. M. Knipe *et al.*, eds. Raven Press Ltd., New York. 1921-1958.

74. Rogers, R. P., J. L. Strominger and S. H. Speck. 1992. Epstein-Barr virus in B lymphocytes: viral gene expression and function in latency. *Adv Cancer Res* 58:1-26.

75. Arrand, J. R. and L. Rymo. 1982. Characterization of the major Epstein-Barr virus-specific RNA in Burkitt lymphoma-derived cells. *J Virol* 41:376-389.

76. Rickinson, A. B. 1990. On the biology of Epstein-Barr virus persistence: a reappraisal. *Adv Exp Med Biol* 278:137-146.

77. Reisman, D., J. Yates and B. Sugden. 1985. A putative origin of replication of plasmids derived from Epstein-Barr virus is composed of two cis-acting components. *Mol Cell Biol* 5:1822.

78. Yates, J., N. Warren, D. Reisman and B. Sugden. 1984. A cis-acting element from the Epstein-Barr virus genome that permits stable replication of recombinant plasmids in latently infected cells. *Proc Natl Acad Sci (USA)* 81:3806.

79. Rawlins, D. R., G. Milman, S. D. Hayward and G. S. Hayward. 1985. Sequence-specific binding of the Epstein-Barr virus nuclear antigen (EBNA1) to clustered sites in the plasmid maintenance region. *Cell* 42:659.

80. Cordier, M., A. Calender, M. Billaud, U. Zimber, C. Rousselet, O. Pavlish, J. Banchereau, T. Tursz, G. Bornkamm and C. M. Lenoir. 1990. Stable transfection of Epstein-Barr virus (EBV) nuclear antigen 2 in lymphoma cells containing the EBV P3HR1 genome induces expression of B-cell activation molecules CD21 and CD23. *J Virol* 64:1002-1013.

81. Wang, F., C. D. Gregory, C. Sample, M. Rowe, D. Liebowitz, R. Murray, A. Rickinson and E. Kieff. 1990. Epstein-Barr virus latent membrane protein (LMP1) and nuclear proteins 2 and 3C are effectors of phenotypic changes in B lymphocytes:EBNA-2 and LMP1 cooperatively induce CD23. *J Virol* 64:2309-2318.

82. Knutson, J. C. 1990. The level of c-fgr RNA is increased by EBNA-2, an Epstein-Barr virus gene required for B-cell immortalization. *J Virol* 64:2530-2536.

83. Abbot, S. D., M. Rowe, K. Cadwallader, A. Risksten, J. Gordon, F. Wang, L. Rymo and A. B. Rickinson. 1990. Epstein-Barr virus nuclear antigen 2 induces expression of the virus-encoded latent membrane protein. *J Virol* 64:2126-2134.

84. Fahraeus, R., A. Jansson, A. Ricksten, A. Sjoblom and L. Rymo. 1990. Epstein-Barr virus-encoded nuclear antigen 2 activates the viral latent membrane protein promoter by modulating the activity of a negative regulatory element. *Proc Natl Acad Sci (USA)* 87:7390-7394.

85. Raab-Traub, N., R. Hood, C. S. Yang, B. Henry and J. S. Pagano. 1983. Epstein-Barr virus transcription in nasopharyngeal carcinoma. *J Virol* 48:580-590.

86. Aubry, J. P., S. Pochon, P. Graber, K. U. Jansen and J. Y. Bonnefoy. 1992. CD21 is a ligand for CD23 and regulates IgE production. *Nature* 358:505-507.

87. Wang, F., S. Tsang, M. Kurilla, J. Cohen and E. Kieff. 1990. Epstein-Barr virus nuclear antigen 2 transactivates the latent membrane protein (LMP1). *J Virol* 64:3407-3416.

88. Boos, H., R. Berger, C. Kuklik-Roos, T. Iftner and N. Mueller-Lantzsch. 1987. Enhancement of Epstein-Barr virus membrane protein (LMP) expression by serum, TPA or n-butyrate in latently infected Raji cells. *Virology* 159:20-30.

89. Contreras-Salazar, B., B. Ehlin-Henriksson, G. Klein and M. G. Masucci. 1990. Upregulation of the Epstein-Barr virus (EBV)-encoded membrane protein LMP in the Burkitt's lymphoma line Daudi after exposure to n-butyrate and after EBV superinfection. *J Virol* 64:5441-5447.

90. Herbst, H., F. Dallenbach, M. Hummel, G. Niedobitek, S. Pileri, Lantzsch,N. Muller and N. Stein. 1991. Epstein-Barr virus latent membrane protein expression in Hodgkin and Reed-Sternberg cells. *Proc Natl Acad Sci (USA)* 88:4766-4770.

91. Hennessy, K., S. Fennewald, M. Hummel, T. Cole and E. Kieff. 1984. A membrane protein encoded by Epstein-Barr virus in latent growth-transforming infection. *Proc Natl Acad Sci (USA)* 81:7201-7211.

92. Hanley, M. R. and T. Jackson. 1987. Substance K receptor : Return of the magnificent seven. *Nature* 329:766-767.

93. Baumann, A., A. Grupe, A. Ackermann and O. Pongs. 1988. Structure of the

voltage-dependent potassium channel is highly conserved from Drosophila to vertebrate central nervous systems. *EMBO J* 7:2457-2463.

94. Tempel, B. L., D. M. Papazian, T. L. Schwarz, Y. N. Jan and L. Y. Jan. 1987. Sequence of a probable potassium channel component encoded at shaker locus of Drosophila. *Science* 237:770-775.

95. Liebowitz, D., R. Kopan, E. Fuchs, J. Sample and E. Kieff. 1987. An Epstein-Barr virus transforming protein associates with vimentin in lymphocytes. *Mol Cell Biol* 7:2299-2308.

96. Liebowitz, D., D. Wang and E. Kieff. 1986. Orientation and patching of the latent infection membrane protein encoded by Epstein-Barr virus. *J Virol* 58:233-237.

97. Martin, J. and B. Sugden. 1991. Transformation by the oncogenic latent membrane protein correlates with its rapid turnover, membrane localization, and cytoskeletal association. *J Virol* 65:3246-3258.

98. Baichwal, V. R. and B. Sugden. 1988. Transformation of Balb/3T3 cells by the BNLF-1 gene of Epstein-Barr virus. *Oncogene* 2:461-467.

99. Wang, D., D. Liebowitz and E. Kieff. 1985. An EBV membrane protein expressed in immortalized lymphocytes transforms established rodent cells. *Cell* 43:831-841.

100. Wang, D., D. Liebowitz and E. Kieff. 1988. The truncated form of the Epstein-Barr virus latent-infection membrane protein expressed in virus replication does not transform rodent fibroblasts. *J Virol* 62:2337-2346.

101. Wang, D., D. Liebowitz, F. Wang, C. Gregory, A. Rickinson, R. Larson, T. Springer and E. Kieff. 1988. Epstein-Barr virus latent infection membrane protein (LMP) alters human B lymphocyte phenotype: deletion of the amino terminus abolishes activity. *J. Virol.* 62:4173-4184.

102. Liebowitz, D. and E. Kieff. 1989. The Epstein-Barr virus latent membrane protein (LMP) induction of B cell activation antigens and membrane patch formation does not require vimentin. *J Virol* 63:4051-4054.

103. Birkenbach, M., D. Liebowitz, F. Wang, J. Sample and E. Kieff. 1989. Epstein-Barr virus infection or the latent infection membrane protein induces vimentin expression. *J Virol* 63:4079-4084.

104. Springer, T. A. 1990. Adhesion receptors of the immune system. *Nature* 346:425-434.

105. Henderson, S., M. Rowe, C. Gregory, D. Croom-Carter, F. Wang, R. Longnecker, E. Kieff and A. Rickinson. 1991. Induction of *bcl-2* expression by Epstein-Barr virus latent membrane protein 1 protects infected B cells from programmed cell death. *Cell* 65:1107-1115.

106. Gregory, C. D., C. Dive, S. Henderson, C. A. Smith, G. T. Williams and J. Gordon. 1991. Activation of Epstein-Barr virus latent genes protects human B cells from death by apoptosis. *Nature* 349:612-614.

107. Dawson, C. W., A. B. Rickinson and L. S. Young. 1990. Epstein-Barr virus latent membrane protein inhibits human epithelial cell differentiation. *Nature* 344:777-780.

108. Fahraeus, R., L. Rymo, J. S. Rhim and G. Klein. 1990. Morphological transformation of human keratinocytes expressing the LMP gene of Epstein-Barr virus. *Nature* 345:447-449.

109. Wilson, J. B., W. Weinberg, R. Johnson, S. Yuspa and A. J. Levine. 1990. Expression of BNLF-1 oncogene of Epstein-Barr virus in the skin of transgenic mice induces hyperplasia and aberrant expression of keratin 6. *Cell* 61:1315-1327.

110. Yao, Q. Y., P. Ogan, M. Rowe, M. Wood and A. B. Rickinson. 1989. Epstein-Barr virus-infected B cells persist in the circulation of acyclovir-treated virus carriers. *Int J Cancer* 43:67-71.

111. Gratama, J. W., M. A. P. Oostterveer, F. E. Zwaan, J. Lepoutre, G. Klein and I. Ernberg. 1988, Eradication of Epstein-Barr virus by allogenic bone marrow transplantation: implications for sites of viral latency. *Proc Natl Acad Sci (USA)* 85:8693-8696.

112. Hudewentz, J., G. W. Bornkamm and H. zur Hausen. 1980. Effect of the diterpene ester TPA on Epstein-Barr virus antigen and DNA synthesis in producer and non-producer cell lines. *Virology* 100:175-178.

113. Biggin, M., M. Bodescot, M. Perricaudet and P. Farrell. 1987. Epstein-Barr virus gene expression in P3HR1-superinfected Raji cells. *J Virol* 61:3120-3132.

114. Miller, G. 1990. The switch between latency and replication of Epstein-Barr virus. *J Infect Dis* 161:833-844.

115. Taylor, N., J. Countryman, C. Rooney, D. Katz and G. Miller. 1989. Expression of the BZLF1 latency disrupting gene differs in standard and defective Epstein-Barr viruses. *J Virol* 63:1721-1728.

116. Farrell, P. J., D. T. Rowe, C. M. Rooney and T. Kouzarides. 1989. Epstein-Barr virus BZLF1 transactivator specifically binds to a consensus AP-1 site and is related to c-fos. *EMBO J* 8:127-132.

117. de The, G. and Y. Zeng. 1986. *Population screening for EBV markers: Toward improvement of nasopharyngeal carcinoma control.* In The Epstein-Barr Virus: Recent Advances. Epstein, M. A. and B. G. Achong, eds. John Wiley and Sons, New York. 237-249.

118. Henle, G., W. Henle and V. Diehl. 1968. Relation of Burkitt's tumor-associated herpes-type virus to infectious mononucleosis. *Proc Natl Acad Sci (USA)* 59:94-101.

119. Jones, J. F., S. Shurin, C. Abramowsky, R. R. Tubbs, C. G. Sciotto, R. Wahl, J. Sands, D. Gottman, B. Katz and J. Sklar. 1988. T-cell lymphomas containing Epstein-Barr viral DNA in patients with chronic Epstein-Barr virus infections. *N Engl J Med* 318:733-741.

120. Weiss, L. M., L. A. Movahed, R. A. Warnke and J. Sklar. 1989. Detection of Epstein-Barr viral genomes in Reed-Sternberg cells of Hodgkin's disease. *N Engl J Med* 320:502-506.

121. Cohen, J. I. 1991. Epstein-Barr virus lymphoproliferative disease associated with acquired immunodeficiency. *Medicine* 70:137-160.

122. Berliner, N. and B. R. Smith. 1991. *The pathobiology of lymphoproliferative disease.* In Hematology: Basic principles and practice. Hoffman, R., E. J. Benz, S. J. Shattil, B. Furie and H. J. Cohen, eds. Churchill Livingstone Inc, New York. 897-912.

123. Korsmeyer, S. J. 1992. *Bcl-2*: a repressor of lymphocyte death. *Immunol Today* 13:285-288.

124. Korsmeyer, S. J. 1992. *Bcl-2* initiates a new category of oncogenes: regulators of cell death. *Blood* 80(4):879-886.

125. Raff, M. C. 1992. Social controls on cell survival and cell death. *Nature* 356:397-400.

126. Wyllie, A. H., J. F. R. Kerr and A. R. Currie. 1980. Cell death: the significance of apoptosis. *Int Rev Cytol* 68:251-306.

127. Alison, M. R. and C. E. Sarraf. 1992. Apoptosis: a gene directed programme of cell death. *J Royal College Physicians* 26:25-35.

128. Duvall, E. and A. H. Wyllie. 1986. Death and the cell. *Immunol Today* 7:115-119.

129. Allen, P. D., S. A. Bustin and A. C. Newland. 1993. The role of apoptosis (programmed cell death) in haemopoiesis and the immune system. *Blood Reviews* 7:63-73.

130. Tsujimoto, Y., L. R. Finger, J. Yunis, P. C. Nowell and C. M. Croce. 1984. Cloning of the chromosome breakpoint of neoplastic B cells with the t(14;18) chromosome translocation. *Science* 226:1097.

131. Tsujimoto, Y., J. Gorham, J. Cossman, E. Jaffe and C. M. Croce. 1985. The t(14;18) translocations involved in B-cell neoplasms result from mistakes in VDJ joining. *Science* 229:1390.

132. Bakhshi, A., J. P. Jensen, P. Goldman, J. J. Wright, O. W. McBride, A. L. Epstein and S. J. Korsmeyer. 1985. Cloning the chromosomal breakpoint of t(14;18) human lymphomas: Clustering around JH on chromosome 14 and near a transcriptional unit on 18. *Cell* 41:889.

133. Cleary, M. L. and J. Sklar. 1985. Nucleotide sequence of t(14;18) chromosomal breakpoint in follicular lymphoma and demonstration of a breakpoint cluster region near a transcriptionally active locus on chromosome 18. *Proc Natl Acad Sci (USA)* 82:7439-7443.

134. Hockenbery, D. M., M. Zutter, W. Hickey, M. Nahm and S. J. Korsmeyer. 1991. *Bcl-2* protein is topographically restricted in tissues characterized by apoptotic cell death. *Proc Natl Acad Sci (USA)* 88:6961-6965.

135. Nunez, G., D. Hockenbery, J. McDonnell, C. M. Sorensen and S. J. Korsmeyer. 1991. *Bcl-2* maintains B cell memory. *Nature* 353:71-73.

136. Tsujimoto, Y. 1989. Overexpression of the human *bcl-2* gene product results in growth enhancement of Epstein-Barr virus-immortalized B cells. *Proc Natl Acad Sci (USA)* 86:1958-1962.

137. Vaux, D. L., S. Cory and J. M. Adams. 1988. *bcl-2* gene promotes haemopoietic cell survival and cooperates with c-myc to immortalize pre-B cells. *Nature* 335:440-442.

138. McDonnell, T. J., N. Deane, F. M. Platt, G. Nunez, U. Jaeger, J. P. McKearn and S. J. Korsmeyer. 1989. *bcl-2*-immunoglobulin transgenic mice demonstrate extended B cell survival and follicular lymphoproliferation. *Cell* 57:79-88.

139. LeBrun, D. P., R. A. Warnke and M. L. Cleary. 1993. Expression of *bcl-2* fetal tissues suggests a role in morphogenesis. *Am J Pathol* 142:743-753.

140. Haldar, S., J. C. Reed, C. Beatty and C. M. Croce. 1990. Role of *bcl-2* in growth factor triggered signal transduction. *Cancer Res* 50:7399-7401.

141. Aisenberg, A. C., B. M. Wilkes and J. O. Jacobson. 1988. The *bcl-2* gene is rearranged in many diffuse B-cell lymphomas. *Blood* 71:969-972.

142. Weiss, L. M., R. A. Warnke, J. Sklar and M. L. Cleary. 1987. Molecular analysis of the t(14;18) chromosomal translocation in malignant lymphomas. *N Engl J Med* 317:1185-1189.

143. Cleary, M. L., N. Galili and J. Sklar. 1986. Detection of a second t(14;18) breakpoint cluster region in human follicular lymphomas. *J Exp Med* 164:315-320.

144. Hua, C., S. Zorn, J. P. Jensen, R. W. Coupland, H. S. Ko, J. J. Wright and A. Bakhshi. 1988. Consequences of the t(14;18) chromosomal translocation in follicular lymphoma: deregulated expression of a chimeric and mutated *bcl-2* gene. *Oncogene Research* 2:263-275.

145. Seto, M., U. Jaeger, R. D. Hockett, W. Graninger, S. Bennett, P. Goldman and S. J. Korsmeyer. 1988. Alternative promoters and exons, somatic mutation and deregulation of the *bcl-2*-Ig fusion gene in lymphoma. *EMBO J* 7:123-131.

146. Kondo, E., S. Nakamura, H. Onoue, Y. Matsuo, T. Yoshino, H. Aoki, K. Hayashi, K. Takahashi, J. Minowada, S. Nomura and T. Akagi. 1992. Detection of *bcl-2* protein and *bcl-2* messenger RNA in normal and neoplastic lymphoid tissues by immunohistochemistry and in situ hybridization. *Blood* 80:2044-2051.

147. Schena, M., L. G. Larsson, D. Gottardi, G. Gaidano, M. Carlsson, K. Nilsson and F. Caligaris-Cappio. 1992. Growth- and differentiation-associated expression of *bcl-2* in B-chronic lymphocytic leukemia cells. *Blood* 79:2981-2989.

148. Shapiro, R. S. 1990. Epstein-Barr virus-associated B-cell lymphoproliferative disorders in immunodeficiency: Meeting the challenge. *J Clin Oncol* 8:371-373.

149. Thomas, J. A., M. J. Allday and D. H. Crawford. 1991. Epstein-Barr virus-associated lymphoproliferative disorders in immunocompromised individuals. *Adv Cancer Res* 57:329-380.

150. Knecht, H., R. Sahli, D. J. L. Joske, E. Bachmann, F. Bachmann, D. Hayoz, B. F. Odermatt and P. Shaw. 1995. *Semiquantitative analysis of Epstein-Barr virus DNA by polymerase chain reaction in clinical samples of lymphoproliferative disorders*. In

Frontiers in Virology 1. Diagnosis of human viruses by polymerase chain reaction technology. Becker, Y. and G. Darai, eds. , .

151. Hamilton-Dutoit, S. J., H. J. Delecluse, M. Raphael, G. Lenoir and G. Pallesen. 1991. Detection of Epstein-Barr virus genomes in AIDS related lymphomas: sensitivity and specificity of in situ hybridization compared with Southern blotting. *J Clin Pathol* 44:676-680.

152. Knecht, H., D. J. L. Joske, E. Bachmann, R. Sahli and B. F. Odermatt. 1992. Significance of the detection of Epstein-Barr virus DNA in lymph notes in patients with Hodgkin's disease. *Leukemia and lymphoma* 8:319-325.

153. Delsol, G., P. Brousset, S. Chittal and F. Rigal-Huguet. 1992. Correlation of the expression of Epstein-Barr virus latent membrane protein and in situ hybridization with biotinylated BamHI-W probes in Hodgkin's disease. *Am J Pathol* 140:247-253.

154. Weiss, L. M., Y. Y Chen, X. F. Liu and D. Shibata. 1991. Epstein-Barr virus and Hodgkin's disease A correlative in situ hybridization and polymerase chain reaction study. *Am J Clin Pathol* 139:1259-1265.

155. Telenti, A., W. F. Marshall and T. F. Smith. 1990. Detection of Epstein-Barr virus by polymerase chain reaction. *J Clin Microbiol* 28:2187-2190.

156. Cohen, J. J. 1993. Programmed cell death and apoptosis in lymphocyte development and function. *Chest* 103 (Suppl):99S-101S.

157. Vaux, D. L. 1993. Toward an understanding of the molecular mechanisms of physiological cell death. *Proc Natl Acad Sci (USA)* 90:786-789.

158. Thorley-Lawson, D. A. and K. P. Mann. 1985. Early events in Epstein-Barr virus infection provide a model for B cell activation. *J Exp Med* 62:45-59.

159. Reed, J. C., Y. Tsujimoto, J. D. Alpers, C. M. Croce and P. C. Nowell. 1987. Regulation of *bcl-2* proto-oncogene expression during normal human lymphocyte proliferation. *Science* 236:1295-1299.

160. Finke, J., R. Fritzen, P. Ternes, P. Trivedi, K. J. Bross, W. Lange, R. Mertelsmann and G. Dolken. 1992. Expression of *bcl-2* in Burkitt's lymphoma cell lines: induction by latent membrane Epstein-Barr virus genes. *Blood* 80:459-469.

161. Wallace, J. M. 1989. Pulmonary infection in human immunodeficiency disease: viral pulmonary infections. *Seminars in Respir Infections* 4:147-154.

162. Lawrence, J. B., C. A. Villnave and R. H. Singer. 1988. Sensitive,

high-resolution chromatin and chromosome mapping in situ: Presence and orientation of 2 closely integrated copies of EBV in a lymphoma line. *Cell* 52:51-61.

163. Klein, G., T. Lindahl and M. Jondal. 1974. Continuous lymphoid cell lines with characteristics of B cells (bone marrow-derived), lacking the Epstein-Barr virus genome and derived from three human lymphomas. *Proc Natl Acad Sci (USA)* 71:3283-3286.

164. Chen, M. L., C. N. Tsai, C. L. Liang, C. H. Shu, C. R. Huang, D. Sulitzeanu, S. T. Liu and Y. S. Chang. 1992. Cloning and characterization of the latent membrane protein (LMP) of a specific Epstein-Barr variant derived from the nasopharyngeal carcinoma in the Taiwanese population. *Oncogene* 7:2131-2140.

165. Higuchi, R., C. H. von Beroldingen, G. F. Sensabaugh and H. A. Erlich. 1988. DNA typing from single hairs. *Nature* 332:543-546.

166. Saiki, R. K., P. S. Walsh, C. H. Levenson and H. A. Erlich. 1986. Genetic analysis of amplified DNA with immobilized sequence-specific oligonucleotide probes. *Proc Natl Acad Sci (USA)* 86:6230-6234.

167. Atkinson, T. and M. Smith. 1984. *Purification of oligonucleotides obtained by small scale (0.2 umole) automated synthesis by gel electrophoresis*. In Oligonucleotide Synthesis: A practical Approach. Gait, M. J., editor. IRL Press, Oxford. 35-81.

168. Impraim, C. C., R. K. Saiki, H. A. Erlich and R. L. Teplitz. 1987. Analysis of DNA extracted from formalin-fixed, paraffin-embedded tissues by enzymatic amplification and hybridization with sequence-specific oligonucleotides. *Biochemical and Biophysical Research Communications* 142:710-716.

169. Cordell, J. L., B. Falini, W. N. Erber, A. K. Ghosh, Z. Abdulaziz, S. MacDonald, K. A. Pulford, H. Stein and D. Y. Mason. 1984. Immunoenzymatic labeling of monoclonal antibodies using immune complexes of alkaline phosphatase and monoclonal anti-alkaline phosphatase (APAAP complex). *J Histochem Cytochem* 32:219-229.

170. Cattoretti, G., P. Stefano, C. Parravicini, M. H. G. Becker, S. Poggi, C. Bifulco, G. Key, L. D'amato, E. Sabattini, E. Feudale, F. Reynolds, J. Gerdes and F. Rilke. 1993. Antigen unmasking on formalin-fixed, paraffin-embedded tissue sections. *J Pathol* 171:83-98.

171. Bolender, R. P., D. M. Hyde and R. T. Dehoff. 1993. Lung morphometry: a new generation of tools and experiments for organ, tissue, cell, and molecular biology. *Am. J. Physiol. (Lung cell Mol Physiol)* 265:L521-L548.

172. Holland, B. S. and M. D. Copenhaver. 1987. An improved sequentially rejective Bonferroni Test procedure. *Biometrics* 43:417-423.

173. Zar, J. H. 1984. *The binomial distribution*. In Biostatistical analysis. Prentice-Hall, Inc.,, New Jersey. 400-402.

174. An, S. F. and K. A. Fleming. 1991. Removal of inhibitor(s) of the polymerase chain reaction from formalin-fixed, paraffin was embedded tissues. *J Clin Pathol* 44:924-927.

175. Cheung, W. Y., A. C. L. Chan, S. L. Loke, G. Srivastava, S. Pittaluga, L. Y. Lim and F. C. S. Ho. 1993. Latent sites of Epstein-Barr virus infection. *Am J Clin Pathol* 100:502-506.

176. Rocchi, G., A. DeFelici, G. Ragona and A. Heinz. 1977. Quantitative evaluation of Epstein-Barr virus-infected mononuclear peripheral blood leukocytes in infectious mononucleosis. *N Engl J Med* 296:132.

177. Murray, R. J., D. Wang, L. S. Young, F. Wang, M. Rowe and E. Kieff. 1988. Epstein-Barr virus-specific cytotoxic T-cell recognition of transfectants expressing the virus-coded latent membrane protein LMP. *J Virol* 62:3747-3755.

178. Hanada, M., D. Della, A. Aiello, E. Stadtmauer and J. C. Reed. 1993. *bcl-2* gene hypomethylation and high-level expression in B-cell chronic lymphocytic leukemia. *Blood* 82:1820-1828.

179. McDonnell, T. J. and S. J. Korsmeyer. 1991. Progression from lymphoid hyperplasia to high grade malignant lymphoma in mice transgenic for the t(14;18). *Nature* 349:254-256.

180. Yunis, J. J., M. G. Mayer, M. A. Arensen, D. P. Aeppli, M. M. Oken and G. Frizzer. 1989. *Bcl-2* and other genomic alterations in the prognosis of large-cell lymphomas. *N Engl J Med* 320:1047-1054.

181. Campos, L., J. P. Roualult, O. Sabido, P. Oriol, N. Roubi, C. Vasselon, E. Archimbaud, J. P. Magaud and D. Guyotat. 1993. High expression of *bcl-2* protein in acute myeloid leukemia cells is associated with poor response to chemotherapy. *Blood* 18:3091-3096.

182. McDonnell, T. J., P. Troncoso, S. M. Brisbay, C. Logothetis, L. W. K. Chung, J. T. Hsieh, S. M. Tu and M. L. Campbell. 1992. Expression of the proto-oncogene *bcl-2* in the prostate and its association with emergence of androgen-independent prostate cancer. *Cancer Res* 52:6940-6944.

183. Lu, Q., R. Poulsom, L. Wong and A. M. Hanby. 1993. *Bcl-2* expression in adult and embryonic non-haematopoietic tissues. *J Pathol* 169:431-437.

184. Tao, Q., G. Srivastava, S. L. Loke and F. C. S. Ho. 1994. Lack of correlation between expression of Epstein-Barr virus (EBV) latent membrane protein and *bcl-2* oncoprotein *in vivo*. *J Clin Pathol* 47:589-591.

185. Schlaifer, D., P. Brousset, M. Attal, P. Massip, C. Payen, B. Marchou, F. Huguet, C. Muller, G. Laurent, J. Pris and G. Delsol. 1994. *bcl-2* proto-oncogene and Epstein-Barr virus latent membrane protein-1 expression in AIDS-related lymphoma. *Histopathol* 25:77-82.

186. Armstrong, A. A., A. Gallagher, A. S. Krajewski, D. B. Jones, B. S. Wilkins, D. E. Onions and R. F. Jarrett. 1992. The expression of the EBV latent membrane protein (LMP1) is independent of CD23 and *bcl-2* in Reed-Sternberg cells in Hodgkin's disease. *Histopathol* 21:72-73.

APPENDIX

I PATIENTS' INFORMATION

Table 4. Lymphocytic Interstitial Pneumonia cases.

Pat #	Age	Sex	Comments/Other diseases
L1	57	M	Acute myocardiac infarction
L2	69	F	Esophageal carcinoma
L3	62	F	Gluten sensitive enteropathy
L4	70	F	Hypothyroidism
L5	50	F	Abdominal and lung lymphoma
L6	50	F	Sjogren's syndrome
L7	42	F	Sjogren's syndrome
L8	55	F	Duodenal ulcer disease
L9	47	M	NA[9]
L10	52	M	NA
L11	59	F	NA
L12	70	M	NA
L13	46	F	Mucosal associated lymphoid tumor of lung.

[9]NA = No clinical information available.

Table 5. Idiopathic Pulmonary Fibrosis cases.

Pat #	Age	Sex	Comments/Other diseases
I21	57	M	Worked in foundry
I22	54	M	Reactive hyperplasia in lymph nodes
I23	71	M	-[10]
I24	64	F	-
I25	65	F	Severe coronary artery disease
I26	46	M	-
I27	62	M	-
I28	57	F	-
I29	61	M	-

[10]- No other comments or complaints.

Table 6. Autopsy cases.

Pat #	Age	Sex	Cause of Death
A11	48	M	Acute myocardial infarction
A12	73	M	Operation of mitral valve
A13	65	M	Intracerebral hemorrhage
A14	76	M	Acute myocardial infarction
A15	68	F	Acute myocardial infarction
A16	74	M	Acute myocardial infarction
A17	60	F	Operation of mitral valve
A18	31	M	Trauma
A19	81	M	Arrhythmia

Table 7. HLA DQα PCR Calibration.

DNA Template (pg)	Band Intensity[11]	Mean ± SD
0	0.498; 0.551; 0.632	0.560 ± 0.067
7	1.650; 2.113; 2.968	2.244 ± 0.669
21	11.420; 9.123; 8.999	9.847 ± 1.363
35	9.590; 10.589; 11.823	10.667 ± 1.119
50	14.174; 13.059; 12.007	13.08 ± 1.084
500	14.588; 16.975; 17.193	16.252 ± 1.445
5000	22.697; 20.497; 23.061	22.085 ± 1.387

$a_0 = 0.4024$
$a_1 = 6.0771$
$r^2 = 0.9536$

Equation of best fit line for band intensity (y) plotted against amount of template DNA (log x):

$$y = 6.0771(\log x) + 0.4024$$

[11]Band intensity was measured as Absorbance at 633 nm relative to the background (=zero).

Table 8. EBV LMP1 PCR Calibration.

DNA Template (pg)	Band Intensity[12]	Mean ± SD
0	0; 0.193; 0.21	0.134 ± 0.117
7	0.271; 0.444; 0.217	0.200 ± 0.08
21	2.160; 2.494; 2.218	2.291 ± 0.178
35	4.412; 3.875; 3.241	3.843 ± 0.586
50	5.313; 5.488; 4.608	5.136 ± 0.466
500	8.544; 8.011; 7.522	8.026 ± 0.511
5000	9.948; 10.524; 10.928	10.467 ± 0.493

$a_0 = -1.9053$
$a_1 = 3.5260$
$r^2 = 0.9759$

Equation of best fit line for band intensity (y) plotted against amount of template DNA (log x):

$$y = 3.5260 (\log x) - 1.9053$$

[12]Band intensity was measured as Absorbance at 633nm relative to the background (=zero).

Table 9. PCR - Amplification of the HLA DQα and EBV LMP1 gene using DNA extracted from LIP, IPF and AC patients.

LIP					IPF					AC				
Pat#[1]	HLA	BI[2,3]	EBV	BI	Pat#	HLA	BI	EBV	BI	Pat#	HLA	BI	EBV	BI
L1	-	NA[4]	NA	NA	I21	-	NA	NA	NA	A31	+	20.8	+	1.9
L2	-	NA	NA	NA	I22	-	NA	NA	NA	A32	+	20.7	-	0
L3	-	NA	NA	NA	I23	-	NA	NA	NA	A33	+	20.9	-	0
L4	-	NA	NA	NA	I24	+	11.7	+	0.5	A34	+	17.4	-	0
L5	-	NA	NA	NA	I25	+	19.4	-	0	A35	+	9.0	+	2.3
L6	-	NA	NA	NA	I26	-	NA	NA	NA	A36	+	12.2	+	1.5
L7	-	NA	NA	NA	I27	-	NA	NA	NA	A37	+	11.5	-	0
L8	-	NA	NA	NA	I28	+	18.5	-	0	A38	+	14.8	+	3.6
L9	-	NA	NA	NA	I29	+	20.1	-	0	A39	+	18.6	+	0.7
L10	-	NA	NA	NA										
L11	-	NA	NA	NA										
L12	-	NA	NA	NA										
L13	+	9.3	-	0										

[1]Pat# = Patient reference number.

[2]BI = Band intensity was measured in arbitrary units using UltroScan densitometer.

[3]In cases where more than one block of lung tissue is available, the average intensity of the PCR product from all blocks is presented.

[4]NA = Not available.

Table 10. Summary of Immunostaining with EBV anti-LMP1.

LIP					IPF					AC				
Pat#	n[1]	f[2]	A[3]	D[4]	Pat#	n	f	A	D	Pat#	n	f	A	D
L1	45	0.67	522.8	0.13	I21	0	0.45	226.7	0	A31	6	0.57	294.2	0.04
L2	259	0.71	771.7	0.47	I22	0	0.8	78.5	0	A32	0	0.57	364.9	0
L3	498	0.77	103.1	3.72	I23	33	0.81	260.8	0.16	A33	0	0.73	256.2	0
L4	223	0.86	350.8	0.74	I24	0	0.84	97.2	0	A34	0	0.49	331.1	0
L5	15	0.67	166.3	0.13	I25	79	0.7	1115	0.1	A35	9	0.75	243.2	0.05
L6	1075	0.67	850.2	1.89	I26	0	0.66	125.4	0	A36	0	0.48	349.1	0
L7	0	0.54	159.4	0	I27	11	0.63	542.4	0.03	A37	0	0.61	266.4	0
L8	274	0.7	212.8	1.84	I28	0	0.43	302.2	0	A38	0	0.43	114.7	0
L9	0	0.88	142.4	0	I29	0	0.91	218.1	0	A39	7	0.23	260.1	0.12
L10	0	0.98	119.9	0										
L11	26	0.89	60.0	0.49										
L12	115	0.91	59.3	2.13										
L13	761	0.6	500.3	2.54										

[1]n = Total number of EBV-infected cells in all blocks available.

[2]f = Mean volume fraction of lung section which is tissue.

[3]A = Total area of slide occupied by lung section based on BioQuant measurement in mm^2.

[4]D = EBV Density is defined as the number of EBV-infected cells per unit area of lung tissue.

Table 11. Summary of Immunostaining data using human anti-*bcl-2* mAb.

LIP			IPF			AC		
Pat#	Sum Cell[1]	Sum Int[2]	Pat#	Sum Cell	Sum Int	Pat#	Sum Cell	Sum Int
L1	21	4	I21	10	8	A31	16	1
L2	27	7	I22	20	2	A32	12	0
L3	17	23	I23	22	5	A33	13	1
L4	15	25	I24	13	12	A34	10	0
L5	22	9	I25	12	3	A35	18	2
L6	30	9	I26	11	11	A36	10	6
L7	11	17	I27	11	8	A37	13	1
L8	22	18	I28	10	28	A38	15	11
L9	19	9	I29	10	17	A39	12	2
L10	11	4						
L11	19	25						
L12	30	5						
L13	26	10						
MeanScore	20.8±6.4	12.7±7.9		13.2±4.5	10.4±8.1		13.2±2.7	2.7±3.6

[1]Sum Cell = Cumulative score for celluarity defined as the sum of scores of 10 randomly selected fields based on cellularity.

[2]Sum Int = Cumulative score for intensity defined as the sum of scores of 10 randomly selected fields based on intensity.

III STATISTICAL ANALYSES

A) EBV status (positive versus negative) among the 3 groups was analyzed using χ^2 statistics corrected for multiple comparison by Tukey-type testing (173)

H_O: Groups (LIP, IPF and AC) are independent of EBV status.
H_A: Groups are NOT independent of EBV status.

$\chi^2 \leq 5.991$ $\therefore p = 0.05$
$\therefore$ Reject H_O, Statistically Significant.

B) EBV Density[1] among the 3 groups was analyzed using single factor ANOVA with sequential rejective Bonferroni procedure to correct for multiple comarisons (172).

H_O: There is no difference in the mean EBV Density among the 3 groups.
H_A: There are differences in the mean EBV Density among the 3 groups.

$F_{0.05(1),2,13} = 9.98130$ $\therefore p \leq 0.003$
$\therefore$ Reject H_O, Statistically Significant.

LIP vs. IPF $F_{0.05(1),1,13} = 11.13872$
$p \leq 0.01$
$\therefore$ Reject H_O, Statistically Significant.

LIP vs. AC $F_{0.05(1),1,13} = 13.39934$
$p \leq 0.01$
$\therefore$ Reject H_O, Statistically Significant.

IPF vs. AC $F_{0.05(1),1,13} = 0.06783$
$p \leq 0.80$
$\therefore$ Do NOT reject H_O, not significant.

[1]Only cases with positive EBV density were logarithmically transformed and evaluated.

C) The mean cumulative cellularity score (based on *bcl-2* immunostaining) among the 3 groups was analyzed using single factor ANOVA and corrected for multiple comparisons using the Bonferroni procedure (172).

H_O: There is no difference in the mean cumulative score for Cellularity among the 3 groups.

H_A: There are differences in the mean cumulative score for Cellularity among the 3 groups.

$F_{0.05(1),2,28} = 8.50854$ $p \leq 0.01$

$\therefore$ Reject H_O, Statistically Significant.

LIP vs. IPF $F_{0.05(1),1,28} = 11.98931$

$p \leq 0.005$

$\therefore$ Reject H_O, Statistically Significant.

LIP vs. AC $F_{0.05(1),1,28} = 11.98931$

$p \leq 0.005$

$\therefore$ Reject H_O, Statistically Significant.

IPF vs. AC $F_{0.05(1),1,28} = 0$

$p = 1.000$

$\therefore$ Do not reject H_O, not significant.

D) The mean cumulative intensity score (based on *bcl-2* immunostaining) among the 3 groups was analyzed using single factor ANOVA and corrected for multiple comparisons using the Bonferroni procedure (172).

H_O: There is no difference in the mean cumulative score for Intensity among the 3 groups.

H_A: There are differences in the mean cumulative score for Intensity among the 3 groups.

$F_{0.05(1),2,28} = 5.67439$ $p \leq 0.009$

$\therefore$ Reject H_O, Statistically Significant.

LIP vs. IPF $F_{0.05(1),1,28} = 0.54802$

$p = 0.46529$

$\therefore$ do not reject H_O, not significant.

LIP vs. AC $F_{0.05(1),1,28} = 10.90128$

$p \leq 0.008$

$\therefore$ Reject H_O, Statistically Significant.

IPF vs. AC $F_{0.05(1),1,28} = 5.55154$

$p \leq 0.05$

$\therefore$ Reject H_O, Statistically Significant.

E) Inter-observer variation of *bcl-2* histological scoring system:
n=100 randomly selected fields; obs A = histological score given by myself (PMK); obs B = histological score give by RGH. Boldface numbers indicate the grade (0, 1, 2 or 3). Plain type numbers within the matrices represent the number of observations corresponding to the grades given by the 2 observers. R_2 is the Pearson $\chi 2$ coefficient of mean square contingency and R_2/R_{2max} is the ratio of the maximal possible value as described in Section 5.3.6.

Cellularity

		obs A		
	Grade	**1**	**2**	**3**
	1	58	1	0
obs B	**2**	20	8	2
	3	1	4	6

R_2 = 0.61
R_2/R_{2max} = 0.75

Intensity

		obs A			
	Grade	**0**	**1**	**2**	**3**
	0	35	12	0	0
obs B	**1**	1	19	7	3
	2	1	1	8	3
	3	0	0	2	8

R_2 = 0.73
R_2/R_{2max} = 0.84

F) Intra-observer variation of *bcl-2* histological scoring system:
n=100 randomly selected fields; run 1 = histological score given in first run (PMK); run 2 = histological score given in second run. Boldface numbers indicate the grade (0,1,2 or 3). Plain type numbers within the matrices represent the number of observations corresponding to the grades given in the 2 runs. R_2 is the Pearson $\chi 2$ coefficient of mean square contingency and R_2/R_{2max} is the ratio of the maximal possible value as described in Section 5.3.6.

Cellularity

		run 1		
	Grade	**1**	**2**	**3**
	1	68	2	0
run 2	**2**	1	14	0
	3	0	2	13

R_2 = 0.78
R_2/R_{2max} = 0.98

Intensity

		run 1			
	Grade	**0**	**1**	**2**	**3**
	0	43	5	0	0
run 2	**1**	1	30	1	0
	2	0	1	12	0
	3	0	0	1	6

R_2 = 0.84
R_2/R_{2max} = 0.97