A STUDY OF THE EFFECTS OF CONSTITUTIVE C-*MYC* EXPRESSION IN EPSTEIN-BARR VIRUS IMMORTALISED B CELL LINES

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ABSTRACT

Endemic Burkitt's lymphoma (BL) is characterised by the presence of Epstein-Barr virus (EBV) and a chromosomal translocation which results in deregulation and constitutive expression of the c-*myc* oncogene. The aim of this study was to examine the role played by constitutive expression of c-*myc* in determining the phenotype of BL cells. In order to mimic the activation of c-*myc*, plasmid constructions, containing a selectable marker and the c-*myc* gene under the control of a strong promoter, were introduced into EBV-immortalised B lymphoblastoid cell lines. Drug resistant cell lines, expressing high levels of c-*myc*, were established and the phenotype of these cells characterised.

This study has demonstrated that constitutive expression of c-*myc* in an EBV immortalised lymphoblastoid cell results in enhanced growth characteristics, including a reduced dependency on exogenous growth factors. However, despite these enhanced growth characteristics, and in contrast to a previous report, constitutive expression of c-*myc* in these cells does not result in a tumorigenic phenotype. This suggests that constitutive expression of c-*myc* and infection with EBV may be insufficient to induce a malignant phenotype in B cells.

Cells transfected with c-*myc* demonstrated decreased homotypic cell adhesion in culture, possibly as a result of decreased expression of the cell adhesion molecule lymphocyte function associated antigen (LFA) -1 on the cell surface. The decrease in LFA-1 expression is a consequence of altered LFA-1 alpha subunit expression, probably as a result of a decreased rate of transcription. LFA-1 is important in B cell/T cell interactions and may be involved in interactions between B cells and other cell types. It is possible that down regulation of this molecule might result in evasion of immunosurveillance, or contribute in some other way to the malignant phenotype.

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LIST OF ABBREVIATIONS

Chemical abbreviations are given in chapter V, section 2.

ACIF	anti-complement immunofluorescence
AIDS	Acquired Immune Deficiency Syndrome
BL	Burkitt's lymphoma
bp	base pairs
CD	cluster of differentiation
cIg	cytoplasmic immunoglobulin
cpm	counts per minute
CsA	cyclosporin A
DOTMA	N[1-2,3-dioleyloxy]propyl
eBL	endemic Burkitt's lymphoma
EA	early antigen
EBNA	Epstein-Barr virus nuclear antigen
EBV	Epstein-Barr virus
ELISA	enzyme linked immunosorbent assay
FACS	fluorescence activated cell sorter
FCS	foetal calf serum
FDC	follicular dendritic cell
g	gram
HIV	Human Immunodeficiency virus
HLA	human leucocyte antigen
ICAM	inter-cellular adhesion molecule
IF	immunofluorescence
Ig	immunoglobulin
IL	interleukin
KDa	kilo-dalton
kb	kilo-base pairs
kV	kilo-volts
LCL	lymphoblastoid cell line
LFA	lymphocyte function-associated antigen
LMP	latent membrane protein
MA	membrane antigen
mA	milliamps
mg	milligram

ml	millilitre
M	molar
mM	millimolar
μFd	micro-faradays
μg	microgram
μl	microlitre
μm	micrometre
mw	molecular weight
ND	not designated
ng	nanogram
NT	not tested
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PBM	peripheral blood mononuclear cells
pg	picogram
PMN	polymorphonuclear cells
RPMI	Rosewell Park Memorial Institute
sBL	sporadic Burkitt's lymphoma
sIg	surface immunoglobulin
TP	terminal protein
U/V	ultra violet
VCA	viral capsid antigen
v/v	volume per volume
WCC	white cell count
w/v	weight per volume

CHAPTER I

INTRODUCTION

1. ONCOGENES

1.1 Introduction

Tumour formation occurs as a result of uncontrolled cell proliferation. Genes involved in promoting or maintaining such proliferation are known as oncogenes, and were first identified from the study of transforming retroviruses. It later became apparent that the transforming genes of retroviruses were originally derived from cellular genes. These cellular genes, or proto-oncogenes, play an important role in normal cellular growth, yet when expressed abnormally, can induce malignant transformation.

With regard to oncogene nomenclature, this study will adopt the terminology of Herrlich and Ponta (1989). The gene is italicized and prefixed to indicate its origin. Thus, c-*myc* refers to the cellular oncogene and v-*myc* to the viral oncogene. The gene products, or onco-proteins, are not italicized and their first letter is upper case (e.g. c-Myc). Cellular oncogenes whose normal expression has been deregulated are referred to as activated.

1.2 Identification of cellular oncogenes

A variety of methods have been used to identify cellular genes - proto-oncogenes - which have the potential to function as oncogenes. The most profitable approach, in terms of numbers of proto-oncogenes identified, has been the characterisation of acutely and non-acutely transforming retroviruses. The two classes of retrovirus differ in their mechanism of transformation. Non-acutely transforming retroviruses induce malignant transformation either by insertion into cellular genes, or their regulatory regions, disrupting normal expression, or by insertion close to the gene thus putting the cellular gene under control of powerful viral regulatory regions (Varmus 1982). A classic example of activation of a normal cellular gene by adjacent retroviral insertion is in Avain leukosis virus (ALV) induced bursal lymphomas, in which the c-*myc* gene is placed under the control of ALV regulatory regions resulting in activation and constitutive expression of the gene (Hayward, *et al.*, 1981). Acutely transforming retroviruses, as their name suggests, cause rapid transformation of cells *in vitro* and *in vivo*, by high level expression of viral oncogene sequences inserted into host cell DNA. Much of the early work on viral oncogenes was carried out using the Rous sarcoma virus (RSV) to transform cells (Martin 1970). It later became

apparent that the viral oncogene (v-*src*) encoded by RSV had a counterpart in normal cells (c-*src*) and that the viral oncogene had arisen by recombination between a retrovirus and the cellular *src* gene (Stehelin *et al.*, 1976; Varmus 1982; Bishop 1987). The 'transduction' of cellular genes into retroviruses, which has enabled over twenty cellular oncogenes to be identified (Bishop 1987; Marshall 1989), results in deregulated expression of the gene. This occurs either as a consequence of placing the gene under the control of viral regulatory regions, or by point mutations and/or deletions within the gene's regulatory regions resulting in normal control of expression being lost (Bishop 1987). The v-*myc* gene of the avian myelocytomatosis virus (MC29; reviewed by Bister and Jansen, 1986) consists of only the two coding exons (II and III) of the c-*myc* gene. The intron between the two exons has been lost, as has the first exon and part of the first intron, regions which contain transcriptional regulatory regions (section 2.2).

A number of other methods have been utilised in the search for potential oncogenes. Analysis of chromosome translocation breakpoints has been used to identify a gene (c-*abl*) thought to be involved in the pathogenesis of chronic myelogenous leukaemia (CML) (de Klein *et al.*, 1982; section 1.4). Characterisation of amplified DNA sequences in tumours has resulted in the discovery of new oncogenes; for example, the identification of amplified sequences with regions of homology to c-*myc* in neuroblastoma led to the discovery of N-*myc* (Schwab *et al.*, 1983; Kohl *et al.*, 1983). Transfection of NIH3T3 fibroblasts with DNA isolated from tumour cells (Shih *et al.*, 1981) has been used to identify a previously uncharacterised member of the *ras* family of oncogenes, N-*ras* (Shimizu *et al.*, 1983).

Using these methods more than 40 human genes with potential transforming activity have been identified (Marshall 1989), although the majority of these genes have not, as yet, been implicated in any human malignancies.

1.3 Role of proto-oncogenes in normal cellular growth

Much of what is now known of the function of proto-oncogenes has been deduced from studying the effects on cells of viral and activated cellular oncogenes. The end-point of oncogene expression is cell proliferation. Normal cell proliferation, or division, is controlled by a group of exogenous polypeptides known as growth factors or mitogens. Growth factors can be either paracrine, that is secreted by other cells, or autocrine, secreted by the host cell. Growth factors exert their effects by triggering cell surface growth factor receptors into sending signals

along second message signal transduction pathways to the nucleus, with the eventual aim of stimulating DNA replication and cell division (reviewed by Sorrentino, 1989). Oncogenes can be divided roughly into two groups; those which generally act within the nucleus, and those which exert their influence outside the nucleus. Examples of nuclear oncogenes are c-*fos* and c-*jun* which, when complexed in a heterodimer, act as a DNA-binding transcription factor (Chiu *et al.*, 1988). Some of the extra-nuclear oncogenes function as growth factors or growth factor receptors. Examples are the c-*fms* gene product which acts as a receptor for macrophage colony stimulating factor-1 (CSF-1) (Sherr *et al.*, 1985), and the cellular gene which encodes the beta chain of platelet derived growth factor (PDGF) from which the *sis* oncogene of simian sarcoma virus is derived (Waterfield *et al.*, 1983). Others appear to function in signal transduction pathways. The protein encoded by c-*src* is a cytoplasmic tyrosine kinase (Collett *et al.*, 1979). Others, notably the *ras* encoded family of proteins localized to the inner surface of the cell membrane, act as guanine nucleotide-binding proteins apparently interacting between growth factor receptors and second message pathways (Hanley and Jackson, 1987).

1.4 Mechanisms of activation

There are many potential mechanisms by which normal cellular proto-oncogene expression could be altered to induce uncontrolled cell proliferation. Alterations at the DNA level, ranging from large deletions and truncations to point mutations, could result in activation of the gene; either through an altered gene product in cases where the lesions occur within the coding region, or through altered expression of an unaltered gene product as a result of disruption of normal regulatory regions. Alternatively, as mentioned earlier, insertion of retroviral regulatory regions adjacent to a proto-oncogene could bring the gene under the control of potent viral regulatory regions (reviewed in Peters, 1989). A good illustration of a point mutation causing activation, is the receptor for the growth factor CSF-1 encoded by c-*fms* (Sherr *et al.*, 1985). A single point mutation in the portion of the gene encoding the extracellular domain of the receptor is sufficient to constitutively activate the receptor's protein tyrosine kinase activity (Roussel *et al.*, 1988). Another widely cited example is that of the human *ras* gene, where single point mutations resulting in single amino acid substitutions are sufficient to induce a transformed phenotype (Reddy *et al.*, 1982; Tabin *et al.*, 1982). An example of a translocation resulting in abnormal transcripts is provided by the t(9;22) translocation in CML. As a result of the translocation the c-*abl* proto-oncogene is translocated to the breakpoint cluster region (*bcr*) of chromosome 22. The role of *bcr* in normal cells is not clear but

normally gives rise to transcripts of 4.0 and 6.5kb (Heisterkamp *et al.*, 1985). Following translocation the fused chimeric *bcr-abl* gene, composed of the 5' end of *bcr* and the 3' end of c-*abl* gives rise to a transcript of approximately 8.5kb (Shtivelman *et al.*, 1985). Since this translocation is invariable in CML it is thought to play an essential role in the development of the tumour, and may be related to the fact that the chimeric gene product (p210) has tyrosine kinase activity, whereas wild-type c-Abl (p150) does not (Clark *et al.*, 1987). Another common mechanism of oncogene activation is amplification. Amplification of c-*myc* (Collins and Groudine, 1982), N-*myc* (Brodeur *et al.*, 1984) and c-*abl* (Collins and Groudine, 1983) have all been reported. With oncogene amplication, at least for N-*myc*, transformation appears to be a consequence of over-production of normal cellular protein, since no sequence differences between amplified and germ-line N-*myc* have been observed (Ibson and Rabbitts, 1988).

It is apparent that the same proto-oncogene can be activated by a variety of different mechanisms. A good example is c-*myc*; in which translocation-induced truncations, point mutations, amplification and proviral insertion have all been implicated in constitutive activation of the gene and tumour development (Cory 1986; Pelicci *et al.*, 1986; Collins and Groudine, 1982; Hayward *et al.*, 1981).

1.5 Oncogenes of DNA viruses

In contrast to retroviruses, the oncogenes of DNA tumour viruses have no known homology with host cell genes. The DNA tumour viruses are a diverse group encoding a variety of oncogenes. Unlike acutely transforming retroviruses they depend on interacting with host cell proteins to assert their tumorigenic effects (reviewed in Lane 1989). In terms of viral strategy the role of the viral oncogene is to facilitate viral replication by subverting host cell mechanisms and inducing cellular DNA replication. One of the major pathways for achieving this is to interact with cellular 'anti-oncogenes' or 'tumour suppressor genes', whose normal function is to prevent indefinite replication. Inactivation of tumour suppressor genes has been implicated in the aetiology of a number of different tumours (Klein, 1987a). An example of viral oncogene products which bind to a cellular tumour suppressor gene are the adenovirus E1A proteins which transform both primary and established cell lines. E1A proteins are potent regulators of both cellular and viral transcription (Moran and Mathews, 1987) and are known to bind to the retinoblastoma (Rb) gene product (p105) (Whyte *et al.*, 1988). Absence of the Rb gene is associated with hereditary

retinoblastoma, a malignancy of children in which inherited heterozygosity at the Rb gene locus predisposes to a tumour of retinoblasts resulting from somatic mutation and loss of expression of the remaining allele (Hansen and Cavenee, 1988). It has been proposed that the function of E1A in transformed cells may be to interact with the Rb gene product, and prevent withdrawal from the cell cycle (Whyte *et al.*, 1988).

2. THE C-*MYC* ONCOGENE

2.1 Introduction

C-*myc* was first identified as the cellular homologue of the viral transforming gene (v-*myc*) of the avian myelocytomatosis virus (MC29) (Sheiness and Bishop 1979). In humans c-*myc* is located on chromosome 8 and is a member of a family of oncogenes, including; N-*myc* and the less well characterised L-*myc*, B-*myc*, P-*myc* and R-*myc* (reviewed by Legouy *et al.*, 1987), all of which share regions of homology, in particular two 70-80bp domains within the second exon of the gene.

2.2 Structure and expression of the gene

The gene is composed of three exons containing one open reading frame (ORF) with a conventional ATG translational start codon close to the 5' end of exon II (Watt *et al.*, 1983). Monoclonal antibodies against c-Myc, however, recognise two proteins of 64 and 67kDa which are found in a variety of cell types (Hann and Eisenman 1984; Ramsay *et al.*, 1984; Eisenman *et al.*, 1985). The two proteins differ only in the N-terminus. Translation of the 64kDa protein is initiated from the ATG codon in exon II, but the larger 67kDa protein initiates from a novel (CTG) codon at the 3' end of exon I (Hann *et al.*, 1988). Myc proteins are known to have very short half lives (about 20 minutes) (Donner *et al.*, 1982; Hann and Eisenman, 1984). A second potential ORF within the first exon has been identified from c-*myc* sequence data derived form human foetal liver cells (Gazin *et al.*, 1984). The same authors also identified two proteins (32 and 58kDa) using antibodies raised against predicted peptide sequences from this ORF (Gazin *et al.*, 1986). These findings are somewhat controversial since other workers sequencing c-*myc* from human foetal liver cells found no ORF within exon I (Watt *et al.*, 1983).

Three promoters have been described in the human c-*myc* gene. P_1 and P_2 are located at the 5' end of exon I with P_1 being located 161bp upstream of P_2 (figure 1; Battey *et al.*, 1983). The 64 and 67kDa proteins are translated from transcripts initiated from these promoters, but there is no evidence for the two proteins being encoded from different promoters, as both are apparently primary translation products from a single mRNA (Hann *et al.*, 1988). In EBV immortalised B cells (section 5.3), in which c-*myc* is normally regulated, there is preferential usage of P_2 relative to P_1 (Taub *et al.*, 1984a). Transcripts initiated from these promoters have very short half lives (10-30 minutes) (Dani *et al.*, 1984; Hann and Eisenman, 1984; Ramsay *et al.*, 1984). The third promoter, P_0, is located approximately 700bp upstream of P_1 (Bentley and Groudine, 1986b). Whilst transcripts initiated at P_0 can be detected, they account for less than 10% of all RNA transcribed from c-*myc* and no protein product has, as yet, been identified (Bentley and Groudine, 1986b). It has been suggested that P_0 might act to express the putative second ORF located in exon I (Gazin *et al.*, 1984, 1986).

Control of transcription is complex, with several positive and negative regulatory regions having been identified (figure 1). A block to transcriptional elongation, which maps to the exon I/intron I border, has been identified in a variety of cell types (Bentley and Groudine, 1986a; Eick and Bornkamm 1986). This results in transcriptional pausing at exon I and is thought to be an important control mechanism. Point mutations and deletions within this region are commonly observed in Burkitt's lymphoma (Pelicci *et al.*, 1986; Cesarman *et al.*, 1987), a tumour in which c-*myc* is constitutively activated (section 4). It has been suggested that these mutations might disrupt normal, premature, termination of transcription resulting in constitutive activation of the gene (Bentley and Groudine, 1986b). Further evidence for this theory was provided by Cesarman *et al* (1987) who found that the block to elongation was commonly absent in BL cell lines with mutations and deletions at the exon I/intron I border.

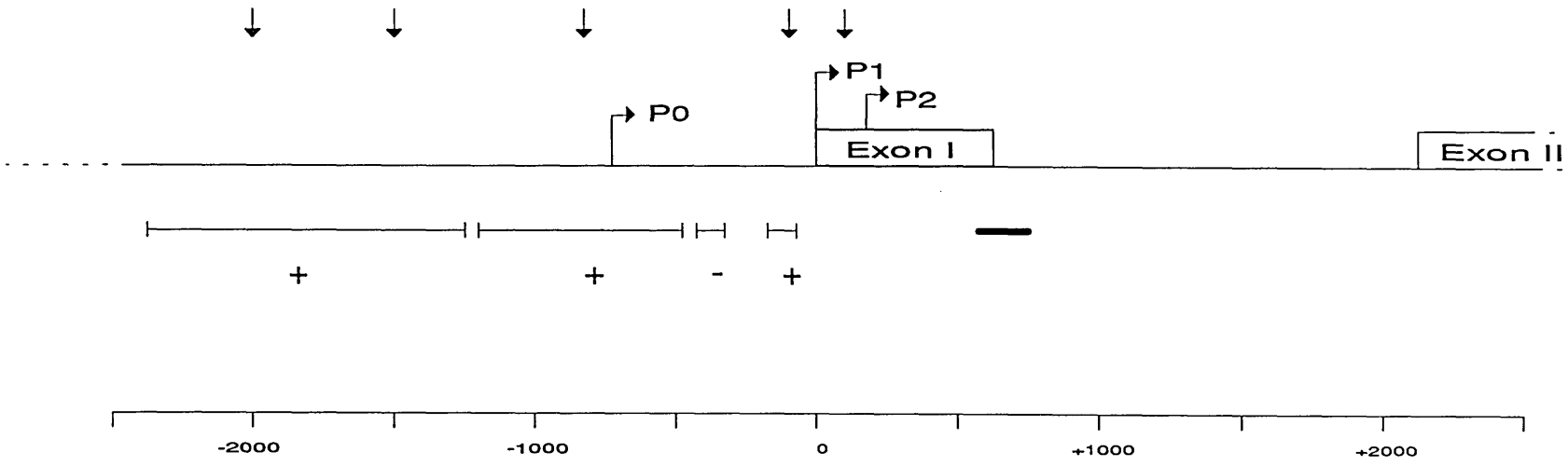
Hay *et al* (1987) identified three positive and one negative regulatory elements located in a 2.3kb region situated directly upstream of P_1. A negative regulatory region (293-353bp upstream of P_1) inhibits expression from both P_1 and P_2 provided the two upstream positive regulatory regions are both absent. These positive regulators appear dominant over the negative regulator, although deletion of the negative regulator increases transcription initiated from P_2. Binding of a factor(s) to a positive regulatory region (101-293 bp upstream of P_1)

Legend: Figure 1

Transcriptional regulatory regions of the c-*myc* gene.

Figure showing known transcriptional regulatory regions of the c-*myc* gene. Horizontal lines indicate regions which positively (+) or negatively (-) influence c-*myc* transcription (Hay *et al.*, 1987). The thick horizontal bar indicates the positition of a block to transcriptional elongation (Bentley and Groudine, 1986a; Eick and Bornkamm, 1986). Vertical arrows indicate the position of known DNase I hypersensitivity sites (Siebenlist *et al.*, 1984). Positions of the three promoters are also indicated (P_0, P_1 and P_3) (Battey *et al.*, 1983; Bentley and Groudine, 1986b).

Figure 1



appears necessary for expression from P_1. Two other regions (257-353 and 1257-2329bp upstream of P_1) appear to act as positive regulatory regions and can act additively to enhance initiation from P_1 and P_2. Sequence similarities to regions which bind known transcription factors suggested that these regions may act as a conventional transcriptional enhancer domain (Hay *et al.*, 1987).

The presence of regulatory regions is supported by the observation of DNase I hypersensitivity sites upstream of the gene during active transcription. These sites disappear when the gene is inactive (Siebenlist *et al.*, 1984). These sites map close to, or within, the three positive regulatory regions (Hay *et al.*, 1987). DNase I hypersensitivity sites have generally been found near to regions of DNA which bind protein and are presumed to occur as a result of changes in chromatin structure (Siebenlist *et al.*, 1984).

Another mechanism by which transcription of c-*myc* is thought to be regulated is that of autosuppression, where high levels of c-Myc protein are thought to suppress transcription of the gene. The idea that c-Myc might autoregulate its own expression was prompted by the observation that the non-translocated allele in BL is transcriptionally silent (Leder *et al.*, 1983; Taub *et al.*, 1984a). In experiments with transgenic mice, possessing one activated c-*myc* allele, the normal allele was also found to be transcriptionally silent (Adams *et al.*, 1985). The same phenomenon has also been observed with N-*myc* which has been shown to regulate both its own transcription and that of c-*myc* (Rosenbaum *et al.*, 1989). In transfection experiments, expression of c-*myc* from a heterologous promoter also resulted in suppression of endogenous c-*myc* transcription (Lombardi *et al.*, 1987; Mango *et al.*, 1989).

There was some debate as to whether the lack of expression of c-*myc* from the untranslocated allele was a consequence of autoregulation. Other mechanisms such as methylation and changes in chromatin structure have been proposed (Dunnick *et al.*, 1985; Nishikura *et al.*, 1988). In some cases high level expression of an exogenous c-*myc* gene was not accompanied by suppression of endogenous c-*myc* (Keath *et al.*, 1984; Coppola and Cole, 1986; Zerlin *et al.*, 1987).

Recent work by Penn and colleagues has clearly demonstrated the phenomenon of auto-regulation by Myc in a wide variety of established and primary rodent fibroblasts (Penn *et al.*, 1990a). They also established that this regulation occurs at the level of transcriptional initiation, and is proportional to the amount of c-

Myc present in the cells. In addition to c-Myc, it is apparent that some other factor is necessary for auto-suppression to occur. High level expression of exogenous Myc in mouse NIH 3T3 fibroblasts did not result in suppression of endogenous transcription. However, fusion of these non-suppressive cells to autosuppression responsive rat fibroblasts (Rat-1) resulted in dominance of the autosuppressive phenotype, with autosuppression of both rat and mouse endogenous c-*myc* (Penn *et al.*, 1990a). The requirement for an additional factor may well explain the various anomalies mentioned above.

In addition to these transcriptional mechanisms c-*myc* anti-sense transcription has been observed upstream of P_1 the role of which is not yet understood (Bentley and Groudine, 1986b). Also regulation of c-*myc* expression is not restricted to transcriptional mechanisms as post-transcriptional controls such as altered mRNA stability have been observed (Dony *et al.*, 1985).

2.3 Function of c-*myc*

Despite being one of the first described and most widely studied oncogene the precise biochemical function of c-*myc* remains unclear. The available evidence, however, points to a direct or indirect role in control of the cell cycle.

In normal cells, c-*myc* expression is mitogen and growth factor responsive. Addition of serum to serum-starved, quiescent fibroblasts results in the rapid, transient, induction of c-*myc* expression (Kelly *et al.*, 1983). In rapidly proliferating cells, stimulated with mitogen, levels of c-*myc* mRNA and protein are maintained throughout the cell cycle (Hann *et al.*, 1985; Thompson *et al.*, 1985; Rabbitts *et al.*, 1985b). Removal of mitogens, or contact with neighbouring cells, results in a rapid decrease in expression of c-*myc* (Rabbitts *et al.*, 1985b; Dean *et al.*, 1986). Overexpression of c-*myc* is known to increase sensitivity to growth factors (Armelin *et al.*, 1984; Kaczmarek *et al.*, 1985; Sorrentino *et al.*, 1986). These data, coupled to the rapid response to external stimuli and rapid turnover of mRNA and protein implicates c-*myc* in growth factor signalling pathways. It has been proposed that c-*myc* might act at a common signal transduction point for a variety of growth factor pathways (Sorrentino *et al.*, 1986).

There is clear evidence for the involvement of c-*myc* in the G_1-S phase of the cell cycle. High level expression of c-*myc* is known to reduce the growth factor requirements for initiation of DNA synthesis (S phase of cell cycle) (Kaczmarek

et al., 1985). Heikkila *et al* (1987) demonstrated that c-*myc* antisense oligonucleotides could inhibit entry of mitogen activated T cells into S phase, and recently Karn *et al* (1989) demonstrated that the length of time a cell spends in G_1 can be reduced by high level expression of an exogenous c-*myc* gene. There is good evidence, however, that c-*myc* does not act directly to control cell cycle, but acts more as a 'competence factor', since overexpression of c-*myc* alone is not sufficient to cause DNA synthesis (Armelin *et al.*, 1984; Kaczmarek *et al.*, 1985). The observation that levels of c-*myc* remain constant throughout the cell cycle (Hann *et al.*, 1985; Rabbitts *et al.*, 1985b) also suggests that there may be other points in the cell cycle at which the gene acts, perhaps by preventing withdrawal from cell cycle, thus promoting continual rounds of cell division in the presence of the necessary growth factors.

C-*myc* may also play an important role in differentiation. In cells where c-*myc* is normally regulated terminal differentiation is preceded by a fall in levels of c-*myc* (Reitsma *et al.*, 1983; Gonda and Metcalfe., 1984; Griep and DeLuca, 1986). Freytag (1988) demonstrated that enforced high level expression of c-*myc* prevented differentiation of a preadipocyte cell line. The block to differentiation was reversible on transfection of c-*myc* antisense mRNA. High level constitutive expression of c-*myc* is also known to prevent dimethyl sulphoxide (DMSO) induced terminal differentiation of murine erythroleukaemia (MEL) cells *in vitro* (Coppola and Cole, 1986; Prochownik and Kukowsa, 1986). Artificial depletion of c-*myc* mRNA in this system resulted in differentiation of MEL cells in response to DMSO (Prochownik *et al.*, 1988). Similar results for F9 murine embryonic teratinocarcinoma cells have also been observed, where retinoic acid induced differentiation was prevented by transfection of a constitutively transcribed c-*myc* gene (Griep and Westphal, 1988). Once again, the block to differentiation was relieved by introduction of c-*myc* antisense transcripts. These findings were challenged by Nishikura *et al* (1990) who reported that enforced expression of c-*myc* in F9 cells was not sufficient to prevent retinoic acid induced differentiation.

Recent work with B cells indicates that expression of c-*myc* correlates with stage of differentiation and proliferation (Nilsson *et al.*, submitted for publication). Chronic lymphocytic leukaemia (CLL) cells represent a synchronised culture of mature resting B cells (section 3.1) which can be induced to proliferate and differentiate *in vitro* by treatment with the phorbol ester, 12-0-tetradecanoyl-phorbol-1,3-acetate (TPA) (Larsson *et al.*, 1987). Nilsson and colleagues demonstrated that c-Myc was not expressed in mature

resting B cells or terminally differentiated plasma blasts but was expressed at high levels in proliferating B-blasts. They propose that the differentiation process in B cells can be split into two stages. The first, involving blast proliferation and initiation of Ig production, is associated with increased expression of c-*myc* in resting B cells which results in transition from G_0 to G_1, and acquisition of responsiveness to growth factors. The second phase, during terminal differentiation to plasma cells, involves down-regulation of c-*myc*, exit from cell cycle and high level production and secretion of Ig and terminal differentiation to plasma cells.

On balance the evidence appears to favour a role for c-*myc* in differentiation, although that role is probably one of increased self-renewal preventing exit from cell cycle and terminal differentiation (Freytag 1988). This is consistent with the findings of Langdon *et al* (1986) who described a relative over-production of pre-B cells in transgenic mice expressing a c-*myc* gene linked to the Ig heavy chain enhancer element. Whilst this hypothesis appears best to explain published observations, the possibility of c-*myc* interacting directly with a differentiation promoting factor cannot be ruled out. For example; c-Myc has structural similarities with MyoD, which is known to promote muscle cell differentiation (Davies *et al.*, 1987). The similarities are discussed in more detail below, but it is not inconceivable that c-Myc might interfere with the function of MyoD-like proteins, thus preventing differentiation.

Despite the considerable evidence for involvement in control of cell cycle, the precise function of c-Myc within the cell is unclear. It is widely assumed that c-Myc will prove to be a sequence-specific DNA-binding transcription factor (Cole *et al.*, 1990). In this respect it would be like many other nuclear proto-oncogenes; for example c-*fos* and c-*jun* which form part of the transcription factor AP-1 (Chiu *et al.*, 1988), and c-*myb* which has recently been identified as a sequence specific transcriptional activator (Weston and Bishop, 1989).

C-Myc is known to bind non-specifically to DNA (Donner *et al.*, 1982; Watt *et al.*, 1985), and the region of the protein involved in non-specific binding has been identified (Dang *et al.*, 1989a). However, since this region can be deleted without any loss of the gene's ability to transform rat embryo fibroblasts (REF) in co-operation with the *ras* oncogene (Land *et al.*, 1983; section 2.4), its biological significance is unclear (Stone *et al.*, 1987; Penn *et al.*, 1990b). There has been considerable speculation that specific binding of c-Myc may be involved in directly promoting DNA replication. Studzinski *et al.* (1986) added

affinity purified antibodies to nuclei isolated from human cells and reported a reversible inhibition of DNA synthesis, implicating c-Myc in promotion of DNA replication. Some doubt was cast on these findings when it was reported that an unidentified inhibitor of DNA polymerases, distinct from the c-Myc specific antibodies, could be purified from the antibody preparation used by Studzynski and colleagues (Gutierrez *et al.*, 1987). These findings were consistent with the observations that other antibodies against c-Myc did not inhibit DNA synthesis, but that antibodies against DNA polymerase alpha were inhibitory (Kaczmarek *et al.*, 1985). Other workers have argued a role for c-Myc in promotion of DNA synthesis on the basis of co-transfection experiments where c-Myc antibodies and an autonomously replicating plasmid were transfected into HL60 cells (Iguchi-Ariga, *et al.*, 1987a). Inhibition of plasmid replication in the presence of c-Myc antibodies, and binding of c-Myc to the plasmid DNA in the absence of antibodies was reported, prompting the authors to suggest that c-Myc may be promoting DNA synthesis by binding to the initiation site of replication (Iguchi-Ariga *et al.*, 1987a). These findings are controversial and could not be repeated by other groups (Gutierrez *et al.*, 1988; Cole 1990). Further work reporting that c-Myc could substitute for the SV40 large T antigen in promoting replication of SV40 based plasmids was also cited as evidence for c-Myc playing a role in promoting DNA replication (Iguchi-Ariga *et al.*, 1987b). Once again these findings are called into question by the data of Gutierrez *et al* (1987) who reported that binding of Myc-specific antibodies to c-Myc did not inhibit SV40 DNA replication. Thus, there remains no clear evidence for c-Myc playing a direct role in DNA replication.

There are considerable structural similarities between c-Myc and two groups of sequence-specific, DNA-binding regulators of gene expression, suggesting that Myc may well eventually prove to be a sequence-specific DNA-binding protein. The two groups are the leucine zipper (LZ) and helix-loop-helix (HLH) proteins.

The helix-loop-helix (HLH) proteins have 10-11 identical amino acids spread over a 40 amino acid region which is predicted to form a helix-loop-helix domain of two amphipathic alpha-helices (Murre *et al.*, 1989). An example of an HLH protein is MyoD; a muscle specific protein which promotes muscle cell differentiation when transfected into muscle stem cells (Davis *et al.*, 1987). MyoD has been shown to interact specifically with the muscle creatine kinase gene enhancer region, and regions homologous to c-*myc* are required for this binding activity (Lassar *et al.*, 1989). The HLH domain itself is not required for binding to DNA but participates in protein-protein dimerization. DNA-binding

properties are assigned to a highly basic region of twenty amino acids adjacent to the HLH domain (Davis *et al.*, 1990). Myc also has a basic region adjacent to a HLH domain but dimerization with other HLH proteins has not been observed (Murre *et al.*, 1989).

LZ proteins also bind DNA via highly basic regions. LZ proteins are characterised by the presence of an amphipathic alpha-helix containing evenly spaced leucines (Landschulz *et al.*, 1988), and it is via this leucine zipper that dimerization with other LZ proteins occurs. Two well characterised examples of LZ proteins are c-Fos and c-Jun which form the AP-1 binding transcription factor (Chiu *et al.*, 1988). Whilst c-Jun can form homodimers the complex is far less stable than c-Fos/c-Jun heterodimers. C-Fos cannot form homodimers (O'Shea *et al.*, 1989). None of the LZ proteins identified to date have been shown to dimerize with c-Myc (Cole 1990). C-Myc homo-oligomers have been observed *in vitro*, with bacterially produced protein (Dang *et al.*, 1989b), but such a phenomenon has not been observed with protein from other sources (Cole 1990). The oligomerization of bacterially produced c-Myc is interesting in that the size of the complex indicates formation of homotetramers. The LZ region is required for dimer formation but not for dimer-dimer complex formation, suggesting that another region, such as the HLH region might be required for dimer-dimer formation (Dang *et al.*, 1989b). It remains to be seen whether this complex is merely an *in vitro* artefact, as yet no such complexes have been isolated from cells. The leucine zipper region of c-Myc is clearly important however, since deletion of leucines within the zipper results in abolition of the transforming and autosuppressive capabilies of v-Myc (Crouch *et al.*, 1990).

Despite the lack of evidence for dimerization with any known HLH or LZ protein the presence of HLH, LZ and highly basic regions within c-Myc strongly suggests a DNA-binding role. The reason why c-Myc has not been shown to bind specifically to DNA may be a consequence of the protein being isolated as a monomer and, like c-Fos, requires to be bound in a heterodimer for DNA binding activity (Gentz *et al.*, 1989). The only evidence for another cellular protein associating with c-Myc was provided by Gillespie and Eisenman (1989) who detected, by chemical cross-linking, association of c-Myc with a large (>500kDa) cellular protein. The identity and function of this protein has not yet been established. More recently, Dr. R. Eisenman, at a meeting in Cold Spring Harbor USA, reported the isolation and sequencing of a completely novel protein which interacts with the HLH and LZ domains of c-*myc* (Marx, J., 1990). These data, if confirmed, provide the first evidence for c-Myc associating

with other cellular proteins via the HLH and/or the LZ regions.

Interestingly, c-Myc contains both HLH and LZ domains, unlike most of the other HLH or LZ proteins which generally possess one, but not both, of the domains. Recently, however, a novel cellular transcription factor (TFE3) with both HLH and LZ domains has been described which binds to the μE3 Ig heavy chain enhancer region (Beckmann *et al.*, 1990).

2.4 Role in tumorigenicity

There is a considerable amount of data on the role played by c-*myc* in the development of tumours. Most of the work has centred on whether activation of c-*myc* alone is sufficient for tumours to occur, or whether activation of other cellular genes is required. There are three main source of data on the tumorigenic potential of c-*myc*; experiments involving infection with v-*myc* carrying retroviruses, transfection of endogenous *myc* genes into cells, and experiments with transgenic mice.

Infection of avian cells, *in vitro*, with v-*myc*-carrying retroviruses is known to result in formation of transformed foci, (Royer-Pokara *et al.*, 1978; Palmieri *et al.*, 1983). However, when *in vitro* transformed fibroblasts were inoculated into nude mice they were found to be non-tumorigenic (Palmieri *et al.*, 1983). Rapp *et al* (1985) examined the *in vivo* tumorigenic potential of v-*raf* and v-*myc* carrying retroviruses. New-born mice infected with the v-*raf* virus developed fibrosarcomas 4-8 weeks after infection. Whilst mice infected with v-*myc* virus did develop B and T cell tumours, they did not arise until at least 9 weeks after infection. A recombinant retrovirus containing both v-*raf* and v-*myc* resulted in the development of T and B cell lymphomas 1-3 weeks after infection. *In vitro* experiments with the same viruses revealed a similar pattern; the v-*myc* virus failed to transform cells and, whilst transformed foci of cells were observed with the v-*raf* virus, the recombinant *raf/myc* virus was much more efficient at transforming cells (Rapp *et al.*, 1985). In contrast Ramsay *et al* (1990) infected chicken embryo fibroblasts *in vitro* and chicks *in vivo* with retroviral vectors carrying either the v-*myc* gene of the MC29 virus or a c-*myc* gene linked to the RSV LTR. They reported transformed colonies *in vitro* and tumours *in vivo* with both *myc* genes. However, whilst the v-*myc* retrovirus induced tumours in 2-3 weeks in all animals examined, the c-*myc* gene induced tumours in only 50% of animals with a latency period of 4-6 weeks.

Transfection of exogenous activated c-*myc* genes into REF cells has been shown to result in an immortalised phenotype and reduced serum dependency, but not a transformed phenotype (Land *et al.*, 1983; Mougneau *et al.*, 1984; Connan *et al.*, 1985; Land *et al.*, 1986; Nicolaiew *et al.*, 1986). However, cells transfected with activated c-*myc* genes are readily transformed by addition of growth factors (Roberts *et al.*, 1985) and phorbol esters (Connan *et al.*, 1985). Whilst transfection of an activated c-*myc* gene into cells has been reported to be insufficient for malignant transformation, co-transfection of c-*myc* and other oncogenes does result in a transformed phenotype. Oncogenes known to co-operate with c-*myc* to induce transformation include *ras* and polyoma middle T antigen (Land *et al.*, 1983; Mougneau *et al.*, 1984).

Balanced against the various reports of transfection of activated c-*myc* genes into cells resulting in an immortalised, non-transformed, phenotype there are several reports of transformed phenotypes in cells transfected with activated c-*myc* genes (Keath *et al.*, 1984; Pellegrini and Basilico, 1986). Thus, from transfection studies, the role of c-*myc* in transformation is unclear. Experiments with transgenic mice, however, clearly indicate that constitutive expression of c-*myc* alone is generally insufficient to induce a neoplastic phenotype.

In initial experiments by Stewart *et al* (1984) transgenic mice, expressing a c-*myc* gene under the control of the mouse mammary tumour virus (MMTV) LTR, were unaffected during early development, but developed mammary adenocarcinomas during their second or third pregnancies. This clearly suggested that at least one additional event was required for tumours to arise. Adams *et al* (1985) generated transgenic mice containing the murine c-*myc* gene coupled to the Igμ enhancer (Eμ). Over 90% of the mice developed monoclonal tumours predominantly of immature and mature B cells. However, the monoclonality of these tumours and the length of time taken for the tumours to develop (up to five months) strongly suggested that other factors were involved. They proposed that the role of c-*myc* was to promote increased cell renewal, thus increasing the chances of a second, tumour-inducing, event occurring. Clear evidence for the role of c-*myc* in promoting self-renewal came from further experiments with Eμ-*myc* transgenic mice, where hyperplasia at the pre-B cell stage was described, suggesting that activation of c-*myc* favours proliferation over differentiation (Langdon *et al.*, 1986). It was also demonstrated that these pre-B cells, expressing the transgene, were not initially tumorigenic, but that they could be transformed, *in vivo* and *in vitro*, by infection with v-H-*ras* or v-*raf* (Alexander *et al.*, 1989; Langdon *et al.*, 1989).

There is some evidence that further events, in addition to activation of *ras* and *myc*, are required for transformation. When *ras* and *myc* transgenic mice were crossed, monoclonal tumours arose in the progeny, suggesting that additional factors are involved in the malignant transformation (Sinn *et al.*, 1987).

2.5 Malignancies associated with c-*myc*

C-*myc* has been implicated in a number of human neoplastic conditions including carcinoma of the breast (Escot *et al.*, 1988) acute T cell leukaemia (Erikson *et al.*, 1986) and small cell lung carcinoma (Little *et al.*, 1983). However, the strongest association between abnormal expression of the gene and a human malignancy is in Burkitt's lymphoma.

3. B CELL DEVELOPMENT

Before describing further the characteristics of BL and the mechanisms by which the tumour might occur, it is important to outline the putative sequence of events in normal B cell development, a simplified overview of which is presented in figure 2.

3.1 B cell differentiation

B cell differentiation (reviewed by Calvert and Cooper, 1988; Ratcliffe and Klaus, 1988; Ling, 1988) can be divided into two phases. The first, in which a stem cell gives rise to a virgin, or mature, B cell, occurs in the bone marrow and is antigen-independent. The second occurs in response to stimulation of mature B cells with antigen. Antigen specific B cell clones are expanded and eventually differentiate into antibody secreting plasma cells or memory B cells.

Cells committed to B cell lineage are derived from pluripotential stem cells in the bone marrow. These 'pro-B' cells, with germ line heavy and light chain immunoglobulin (Ig) genes, give rise to large dividing pre-B cells. These cells re-arrange heavy chain variable (V_H), diversity (D), joining (J) and constant (C) gene segments to express cytoplasmic μ heavy chains. No surface Ig (sIg) or light chain expression is seen. These cells differentiate to small non-dividing

Legend: Figure 2

B cell differentiation and expression of cell surface antigens.

Figure showing putative B cell differentiation pathway and expression of B cell surface molecules at the various stages of differentiation. Figure 2a shows the early, antigen independent stages of differentiation and primary activation of B cells in the extrafollicular regions of secondary lymphoid tissue. Figure 2b shows the secondary phase of B cell activation in which occurs when circulating memory B cells encounter antigen on the follicular dendritic cells (FDC) of the lymphoid germinal centres. Ig - immunoglobulin; c - cytoplasmic; s - surface; V - variable; D - diverse; J - joining; +Ag - encounters antigen; -Ag - does not encounter antigen.

Figure 2a

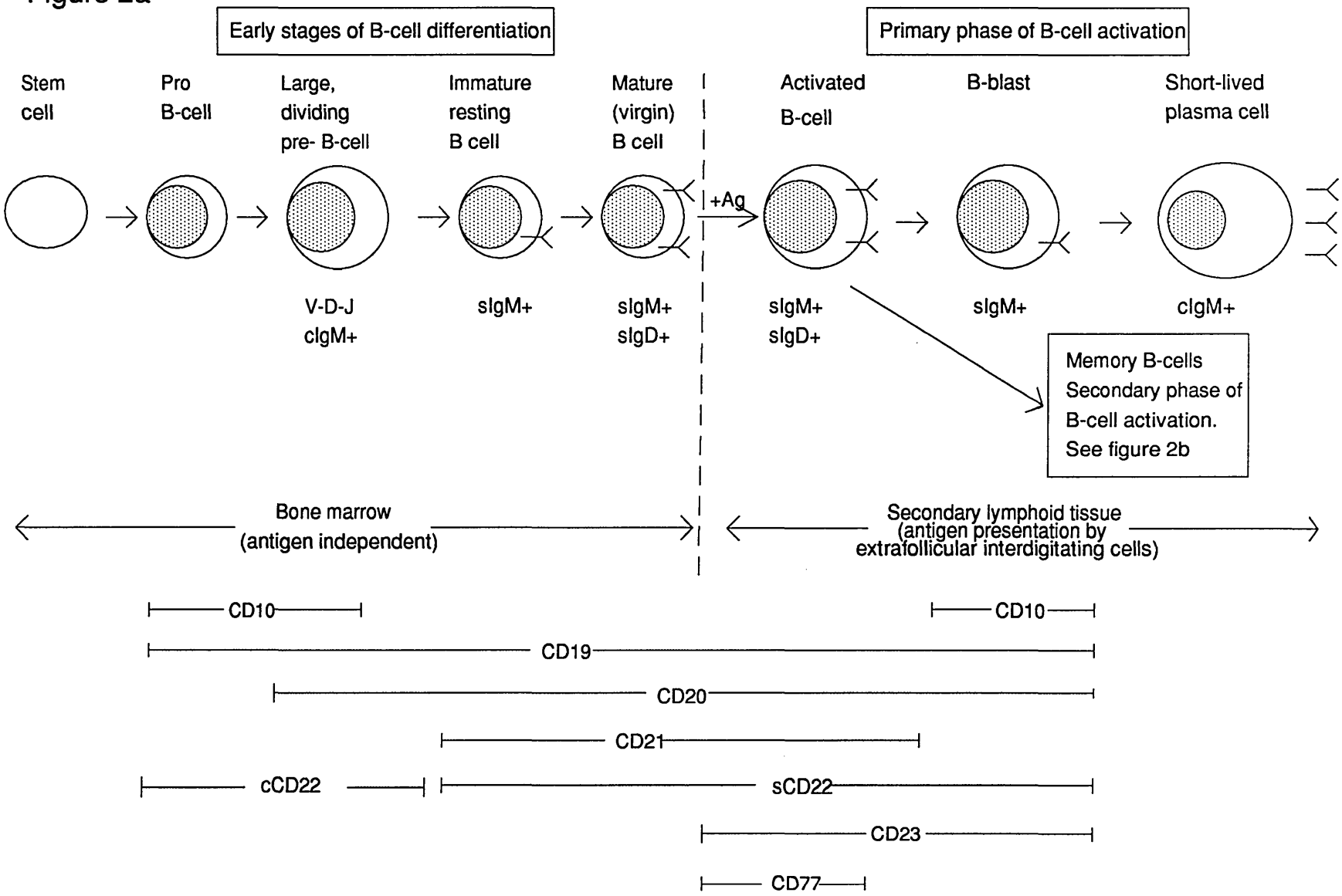
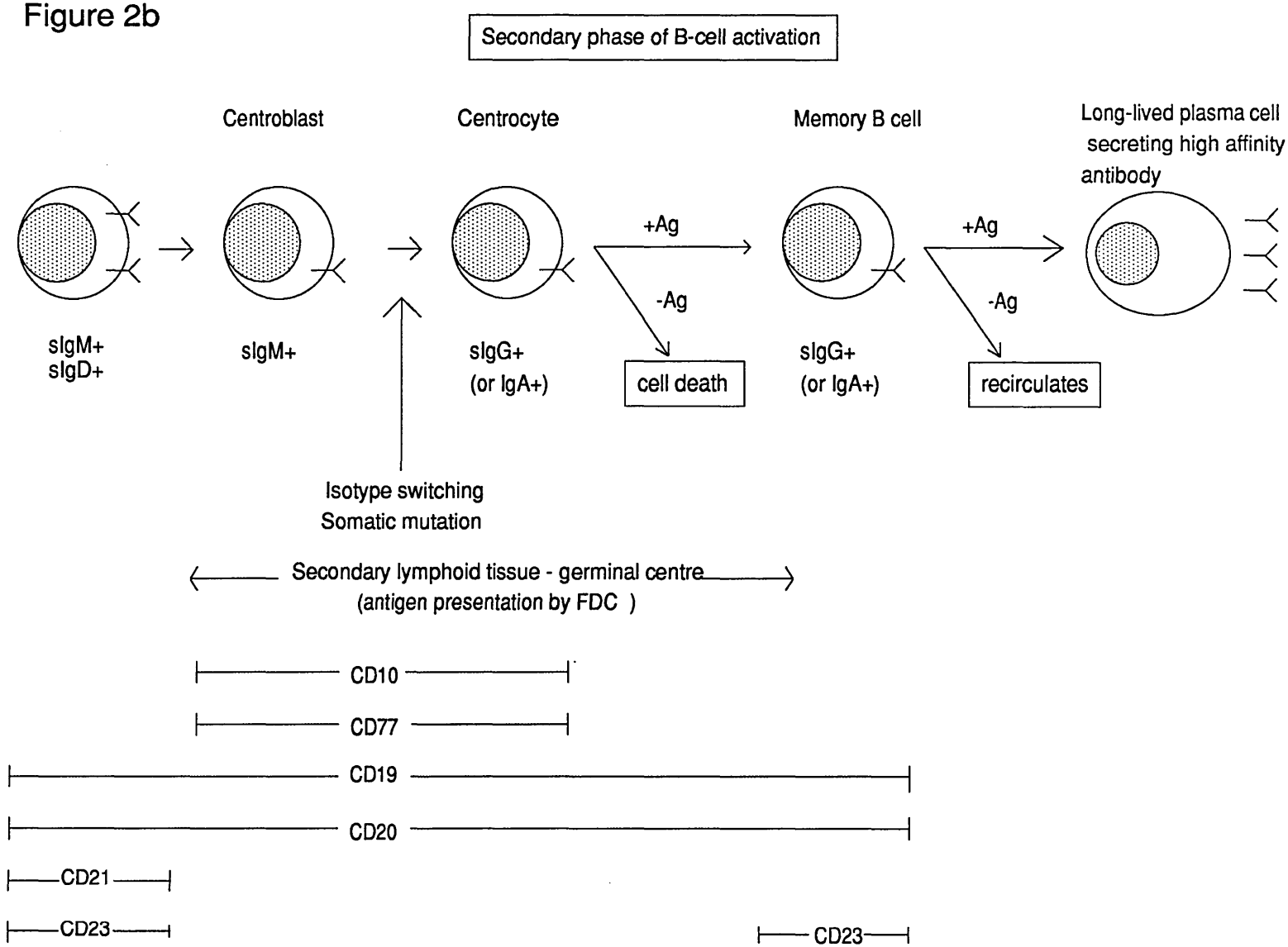

Early stages of B-cell differentiation
Primary phase of B-cell activation
Stem cell
Pro B-cell
Large, dividing pre- B-cell
Immature resting B cell
Mature (virgin) B cell
Activated B-cell
B-blast
Short-lived plasma cell
+Ag
V-D-J cIgM+
sIgM+
sIgM+ sIgD+
sIgM+ sIgD+
sIgM+
cIgM+
Memory B-cells Secondary phase of B-cell activation. See figure 2b
Bone marrow (antigen independent)
Secondary lymphoid tissue (antigen presentation by extrafollicular interdigitating cells)
CD10
CD10
CD19
CD20
CD21
cCD22
sCD22
CD23
CD77

Figure 2b


Secondary phase of B-cell activation
Centroblast
Centrocyte
Memory B cell
Long-lived plasma cell secreting high affinity antibody
+Ag
-Ag
+Ag
-Ag
sIgM+ sIgD+
sIgM+
sIgG+ (or IgA+)
cell death
sIgG+ (or IgA+)
recirculates
Isotype switching
Somatic mutation
Secondary lymphoid tissue - germinal centre
(antigen presentation by FDC)
CD10
CD77
CD19
CD20
CD21
CD23
CD23

pre-B cells, re-arrange light chain V and J gene segments to produce functional kappa or lambda light chains, and express surface IgM. On differentiating further, IgD is also expressed on the cell surface. All these stages are independent of antigen stimulus and it is at this stage that the B cells leave the bone marrow as small mature virgin B cells able to respond to antigenic stimulus.

B cell activation, in response to interaction of antigen with Ig receptors on the B cell surface, is thought to be divided into two phases (MacLennan and Gray, 1986). During the early, or primary, phase circulating mature virgin B cells are activated by exposure to antigen, presented by extra-follicular interdigitating cells, in the extrafollicular regions of secondary lymphoid tissue. As a consequence, cell size increases, and these cells (B-blasts) proliferate to form either circulating memory B cells or short-lived antibody producing plasma cells. The antibody produced is generally IgM, and of relatively low affinity. During the secondary, or late, phase of B cell activation, mature memory B cells circulate through the extra-follicular regions of secondary lymphoid tissue. If they encounter antigen they differentiate into short lived plasma cells or re-circulate as memory B cells. If they are not activated by antigen they migrate to the follicular dendritic cells (FDC) of the germinal centre. Activation by antigen, presented on the FDC, results in formation of one of three cell types: long-lived antibody producing plasma cells, memory B cells or centroblasts. As centroblasts proliferate to give rise to centrocytes, somatic mutation of Ig V region genes and isotype switching is thought to occur (Berek and Milstein, 1987; McClennan and Gray, 1986). Somatic mutation of V regions is regarded as a 'fine-tuning' stage which gives rise to high affinity antibody producing B cells. Exposure to antigen selects high affinity antibody bearing B cells, and recent evidence suggests that non-selected centrocytes rapidly die (Liu *et al.*, 1989). The end result is a long-lived memory B cell with the capacity, on encountering the relevant antigen, for rapid production of plasma cells producing high affinity IgG, or IgA, class antibody.

3.2 Expression of B cell surface antigens

B cell development is characterised by the expression of a number of B cell surface molecules (reviewed by Ling *et al.*, 1987; Dorken *et al.*, 1989; Schwaling and Stein, 1989). Some of these molecules are expressed on virtually all B cells (referred to as pan B cell markers), whilst others have a far more restricted range of expression (figure 2). Of all the B cell markers described, CD19 is the

most broadly expressed, being present on B cells from the pro-B cell stage right through to plasma cells, where expression is lost. CD22 is another pan-B cell marker, although expression is confined to the cytoplasm during the pro and pre-B cells. CD22 is first expressed on the cell surface at the immature resting B cell stage and is lost during terminal differentiation to the plasma cell stage. CD20 is first detectable on pre-B cells and is expressed during differentiation to B-blasts but is lost on terminal differentiation to plasma cells. CD21 is the receptor for the C3d component of complement and also for Epstein-Barr virus (EBV) (section 5.3). It is expressed on mature resting B cells but is lost during the early stages of B cell activation. High level of expression is seen on the FDC, mantle and marginal cells of secondary lymphoid tissue, but not on germinal centre B cells. CD23 is expressed at low levels on mature circulating B cells but not on sIgM and sIgD positive B cells of the bone marrow. Expression of the molecule is increased on activation by antigen. CD23 is a low affinity receptor for IgE and has also been reported as a receptor for low molecular weight B cell growth factor (BCGF) (Gordon *et al.*, 1986). The role of CD23 as the receptor for BCGF is controversial since CD23 negative B cells can respond to low molecular weight BCGF (Vasquez *et al.*, 1988; Azim *et al.*, 1990). The extra-cellular portion of the molecule is cleaved and has been reported as having autocrine growth factor activity (Swendeman and Thorley-Lawson, 1987). CD10, formerly known as common acute lymphoblastic leukaemia antigen (cALLa), is expressed early in B cell differentiation, on pro and pre-B cells, and on B-blast cells, but not on activated cells. It is also found on germinal centre centroblasts in secondary lymphoid tissue. Another molecule expressed on centroblasts is CD77 (Burkitt's lymphoma-associated antigen - BLA) (Murray *et al.*, 1985; Gregory *et al.*, 1987). This molecule, however, is not generally expressed in other stages of B cell development. Other molecules found on activated B cells, but not resting mature B cells, include CD30 (Ki1) and CDw70 (Ki24).

3.3 Expression of cell adhesion molecules

Cell adhesion molecules are of fundamental importance in immune cell interactions (reviewed by Springer *et al.*, 1987; Kishimoto *et al.*, 1989; Springer, 1990). These molecules include; lymphocyte function-associated antigen-1 (LFA-1)(CD11a/CD18), LFA-3 (CD58) and the inter-cellular adhesion molecule-1 (ICAM-1) (CD54). LFA-1 is a member of the β-2 leucocyte integrin family and, in common with other members of the family, Mac-1 and p150,95, is a heterodimer composed of a unique alpha subunit (CD11a) non-covalently associated with a beta (CD18) subunit common to Mac-1 and p150,95 (Sanchez-Madrid *et al.*, 1982). The two subunits undergo post-translational modification

and assembly into the functional LFA-1 molecule in the golgi apparatus prior to transport to the cell surface (Sanchez-Madrid *et al.*, 1983; Kishimoto *et al.*, 1989). LFA-3 (CD58) (Sanchez-Madrid *et al.*, 1982) and ICAM-1 (CD54) (Rothlein *et al.*, 1986) are both members of the Ig gene superfamily. Adhesion between cytotoxic T cells and their target cells (for example EBV infected B cells) is known to be mediated via two pathways; interaction between LFA-1 and its ligand ICAM-1 and interaction between LFA-3 and its ligand the T cell specific molecule CD2 (Shaw *et al.*, 1986). Homotypic B cell adhesion of EBV infected B cells is also mediated, at least in part, through the LFA-1/ICAM-1 pathway (Rothlein *et al.*, 1986). Recently, a second ligand for LFA-1 - ICAM-2 - has also been identified (Staunton *et al.*, 1982).

Expression of LFA-1 is restricted to leucocytes, and is first detected on pre-B cells (Krensky *et al.*, 1983; Campana *et al.*, 1986). LFA-3 has a broad distribution and is expressed on most endothelial, epithelial and haematopoietic cells (Krensky *et al.*, 1983). ICAM-1 is expressed on vascular endothelial cells, thymic epithelium, activated T cells, macrophages, germinal centre FDC and B cells (Dustin *et al.*, 1986). Low level expression of ICAM-1 is found on mature circulating B cells, but expression is rapidly increased following activation of B cells (Dustin *et al.*, 1986).

4. BURKITT'S LYMPHOMA

4.1 Introduction

Burkitt's lymphoma (BL) is a monoclonal B cell tumour, characterised by the presence of a chromosome translocation involving the c-*myc* proto-oncogene on chromosome 8. It is classified as an undifferentiated high-grade malignant monoclonal lymphoma of small non-cleaved B cells (Rosenberg *et al.*, 1982). It was first recognised as a distinct clinical and pathological entity by Denis Burkitt, after whom the tumour is named. He described a relatively common malignancy of African children between the ages of 2 and 14, commonly presenting as a tumour of the jaw (Burkitt, 1958), which was subsequently identified as a lymphoma (O'Conor and Davies, 1960).

4.2 Endemic BL (eBL)

BL is endemic in certain parts of the world, most notably Central Africa and Papua New Guinea, and areas of high incidence correspond closely with those where malaria is holoendemic, suggesting a role for malaria as a co-factor (section 4.9). In central Africa, BL is the commonest childhood tumour, occurring at an incidence of 5/100000 in children under 14 years (Geser *et al.*, 1983). There is a close association with a human herpes virus, Epstein-Barr virus (EBV) (section 5), which is detected in 96% of BL cases in endemic areas (Geser *et al.*, 1983).

Nkrumah and Olweny (1985), in a review of the clinical aspects of endemic BL from data collected over 15 years in central Africa, noted that the commonest sites of presentation were: the jaw (59-72% of cases); abdomen (56-59%); central nervous system (CNS) (28-38%). Less frequently, tumours in the thyroid, breast, salivary glands, bone and testes were observed. Only very rarely were peripheral lymph nodes involved. Abdominal lesions were commonly found in the ovaries of female patients and other common abdominal sites were kidneys, liver and gastrointestinal tract. Bone marrow involvement was observed in a minority (13%) of patients.

4.3 Sporadic BL (sBL)

BL also occurs, at low incidence (0.1-0.2/100000), throughout the world (Magrath and Sariban, 1985). This form of the tumour is known as sporadic BL and differs from endemic BL in a number of features. It is not confined to children, with an age range of 1 - 87 years, although over 50% of patients are aged under 15 years (Levine *et al.*, 1985). In contrast to the endemic form of the tumour, EBV is only found in a minority (15%) of sporadic BL cases (Lenoir, 1986). The incidence of EBV negative BL in regions where BL is endemic correlates with the incidence of sBL worldwide (Lenoir, 1986).

Sites of clinical presentation differ from those commonly seen in eBL. In a National Cancer Institute study of sporadic BL in the USA the commonest sites of tumour were; the abdomen (91% of cases), bone marrow (22%), CNS (14%). In contrast to endemic BL, tumours of the jaw were rare, present in only 9% of cases (Magrath and Sariban, 1985). In addition to the clinical and epidemiological differences between the two forms of the tumour, there are also genetic differences (see section 4.5).

4.4 BL in individuals infected with human immunodeficiency virus

It has been recognised for some time that patients infected with the human immunodeficiency virus (HIV) have a increased risk of developing lymphoproliferative disease (Ziegler *et al.*, 1982, 1984). 1-5% of individuals infected with HIV have been reported to develop non-Hodgkin's lymphoma (NHL), but this figure may well prove to be an underestimate (Ernberg 1989). Two major groups of lymphoproliferative disease have been described (Kalter *et al.*, 1985). The first occurs mainly in patients with advanced AIDS, in whom T cell function is profoundly impaired. The lymphoproliferative disease that occurs in these patients closely resembles the large cell lymphomas seen in patients undergoing long term immunosuppressive therapy, usually following organ transplantation (discussed in section 5.7.2). The second type of lymphoproliferative disease occurs in patients with relatively early stage AIDS, with a less pronounced T cell dysfunction. The usual site of presentation is the abdomen and the tumours histologically resemble BL. It has been estimated that these account for 25-50% of all lymphomas in AIDS patients, and the risk of developing BL is several thousand fold higher in HIV individuals compared to normal healthy individuals (Ernberg, 1989). Most of the tumours are more characteristic of the sporadic form of BL, in terms of translocations and clinical features (Subar *et al.*, 1988; Neri *et al.*, 1988). The majority of HIV associated BL are EBV negative, although EBV-positive tumours resembling sBL have been described (Subar *et al.*, 1988).

4.5 The c-*myc* translocation

Virtually all described cases of BL are characterised by one of three unilateral, reciprocal chromosome translocations involving the c-*myc* proto-oncogene on chromosome 8. The commonest translocation, t(8;14)(q24;q32) results in translocation of the c-*myc* gene on chromosome 8 to the Ig heavy chain loci on chromosome 14 (Manolov and Manolova, 1972; Zech *et al.*, 1976). Less common are the translocations involving c-*myc* on chromosome 8 and the kappa Ig light chain locus on chromosome 2 [t(2;8)(p11;q24)] (Erikson *et al.*, 1983) or the lambda Ig light chain locus on chromosome 22 [t(8;22)(p24;q11)] (Croce *et al.*, 1983). The relative incidences of the t(8;14), t(2;8) and t(8;22) translocations have been calculated as 76, 8 and 16% respectively (Lenoir, 1986).

Figure 3 represents diagramatically the regions where the chromosome breakpoints are known to occur within the c-*myc* and Ig genes. Molecular cloning and analysis of breakpoints has revealed particular regions of the Ig loci

Legend: Figure 3

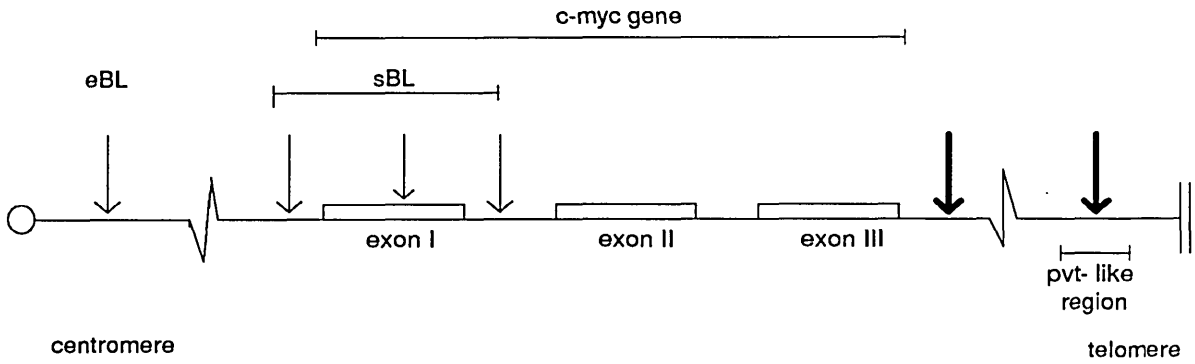
Chromosome translocation breakpoints with respect to c-*myc* on chromosome 8 and IgH on chromosome 14

A: The c-*myc* gene on chromosome 8 with the regions involved in translocations indicated. The thin arrows indicate the t(8;14) translocation breakpoint regions and the thick arrows the t(2;8) or t(8;22) translocations. Arrows are not intended to indicate specific breakpoints but rather regions of DNA commonly involved in translocations. The broken line indicates distances of greater than 10kb. The locations of the *pvt*-like region and the regions commonly involved in translocations in sporadic (sBL) and endemic (eBL) BL translocations are indicated. Diagram not to scale.

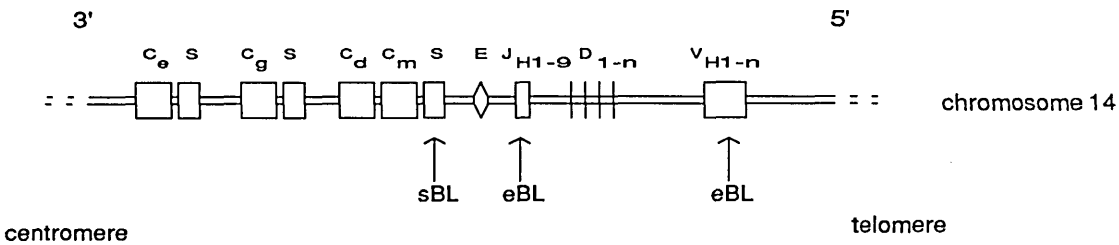
B: Structure of the Ig heavy chain gene situtated on chromosome 14. Regions involved in BL translocations are indicated. The diagram, which is not to scale, is adapted from Flanagan (1988). C - constant; S - switch; E - enhancer; J - joining; D - diversity; V - variable; m - mu; d - delta; g - gamma; e - epsilon.

Figure 3

A:


c-myc gene
eBL
sBL
exon I
exon II
exon III
pvt- like
region
centromere
telomere

B:


3'
5'
C_e S C_g S C_d C_m S E J_{H1-9} D_{1-n} V_{H1-n}
chromosome 14
sBL
eBL
eBL
centromere
telomere

which are frequently involved in translocations, but much more heterogeneity within the c-*myc* gene locus. In terms of the IgH locus, the (8;14) translocations can be broadly divided into two groups. Those which occur in the J region (Haluska *et al.*, 1986; Neri *et al.*, 1988) are predominantly eBLs, and are presumed to arise during normal rearrangment of the heavy chain gene segments (V, D, and J). V-D-J recombination is mediated by the V-D-J recombinase enzyme (Tonegawa, 1983) and it is thought that mistakes by this recombinase are responsible for the translocation (Haluska *et al.*, 1986). Occasional t(8;14) breakpoints are also seen which occur within the V_H or D_H regions (Erikson *et al.*, 1982; Haluska, *et al.*, 1987). The majority of sBL involve the switch region of the IgH chain locus (Gelmann *et al.*, 1983; Showe *et al.*, 1985; Neri *et al.*, 1988). This region is involved in isotype class switching which results in a change from production of IgM to IgG or IgA. Similar to V-D-J rearrangements, a switch recombinase enzyme is thought to be involved in this mechanism (Vose and Holmes, 1988).

The breakpoints involved in the variant t(2;8) and t(8;22) translocations are less well documented, but have been reported involving the V and J regions of the kappa light chain gene, and there is evidence for involvement of V-J recombinase in these translocations (Erikson *et al.*, 1983; Taub *et al.*, 1984b; Hartl and Lipp, 1987). As a consequence of the variant translocations the kappa or lambda constant regions are translocated to the 3' end of the c-*myc* gene on chromosome 14.

It has been suggested that the differences in the regions of Ig loci involved in the translocations between the sporadic and endemic forms indicates that the two arise at different stages in B cell differentiation (Croce and Nowell, 1985; Pelicci *et al.*, 1986; Haluska *et al.*, 1986). V-D-J recombination occurs in pre B cells, whereas isotype switching occurs in more mature B cells (section 3.1). This hypothesis is supported by the fact that eBL cells express cytoplasmic or surface Ig, but do not generally secrete Ig. In sBL cells, however, IgM is often secreted, indicating a later stage B cell (Benjamin *et al.*, 1982; Pelicci *et al.*, 1986).

With regard to c-*myc*, if the (8;14) translocation is considered then two distinct patterns emerge. In sBL the breakpoints are generally located either within the first exon or intron of c-*myc* or close to the 5' end of the gene (Pelicci *et al.*, 1986; Neri *et al.*, 1988), in regions known to be important in regulation of c-*myc* (section 2.2). In contrast, the breakpoints in eBL, involving t(8;14) occur some

considerable distance upstream of the gene. Pelicci *et al* (1986) examined 18 cases of eBL and 14 cases of sBL. In all of the sBL, but only 2 eBL, the chromosome breakpoints occurred within or close to the gene. In the remaining 16 eBL cases the breakpoint was more than 5kb upstream of the gene. The same authors, however, noted restriction enzyme polymorphisms close to or within the gene in 10/10 eBL but only 1/10 sBL. Further studies confirmed the frequent occurrence of point mutations and deletions in a region spanning the junction of exon 1 and intron 1 of c-*myc* (Cesarman *et al.*, 1987; Morse *et al.*, 1989). These lesions occur within, or close to, a c-*myc* transcriptional regulatory region (Bentley and Groudine, 1986; Eick and Bornkamm, 1986; section 2.2).

In the variant (2;8) and (8;22) translocations the breakpoints occur downstream of the gene, either close to the 3' end (Showe and Croce, 1987) or some considerable distance from the gene; more than 50kb away in some cases (Mengle-Gaw and Rabbitts, 1986; Sun *et al.*, 1986). A cluster of t(2;8) breakpoints has been observed to occur within a downstream region, called *PVT*, homologous to the murine plasmacytoma (MPC) variant translocation region (*pvt*-1) (Graham and Adams, 1986). *Pvt*-1 is a 4kb region located 75kb downstream of the murine c-*myc* locus on chromosome 15 (Banjeree *et al.*, 1985). The locus is involved in a variant translocation (6;15) found in MPC. MPC is considered analogous to BL in humans, involving juxtaposition of the murine c-*myc* gene on chromosome 15 with the IgH gene on chromosome 12 or, more rarely, the (6;15) translocation involving the murine Ig kappa gene (Klein 1983). In humans *PVT* is transcribed into a number of different size mRNAs in normal cells, but no proteins have been described. In BL, chimeric transcripts derived from the first exon of *PVT* fused to the the Ig light chain genes have been described, but as in normal cells no protein has been detected suggesting that abnormal expression of this gene does not contibute to the pathogenicity of BL (Shtivelman and Bishop, 1990).

4.6 Activation of c-*myc* in Burkitt's lymphoma

Various transcriptional and post-transcriptional mechanisms for activation of c-*myc* in BL have been proposed including; altered promoter usage (Taub *et al.*, 1984b), insertion of Ig enhancer elements (Hayday *et al.* 1984) and increased mRNA stability (Eick *et al.*, 1985; Piechaczyk *et al.*, 1985; Rabbitts *et al.*, 1985a). If frequency of occurrence is important then the mechanism which appears most likely to play an important role in activation of c-*myc* is the disruption of normal negative transcriptional regulatory regions (discussed in

section 2.2) either by point mutations and deletions or by translocational truncation (Pelicci *et al.*, 1986; Cesarman *et al.*, 1987). This does not rule out the possibility that a number of different mechanisms might converge in the same endpoint of c-*myc* activation.

If point mutations and deletions in regulatory regions are the vital step in activation of c-*myc*, there remains the question of what the translocation contributes to activation of c-*myc*. Its apparently invariable presence in BL implies that it is essential for the development of the tumour yet in some cases the translocation occurs some considerable distance away from the gene. Hayday *et al.* (1984) proposed that the t(8;14) translocation, in which c-*myc* and Ig heavy chain loci are juxtaposed (section 4.5), might bring the c-*myc* gene under the control of the constitutively active Ig enhancer region. Whilst this is clearly possible for some BL it cannot be a universal mechanism since in many BL the Ig enhancer elements and c-*myc* are situated on different chromosomes following translocation (Battey *et al.*, 1983; Rabbitts *et al.*, 1983).

4.7 Evidence linking EBV to endemic BL

The two characteristic features of eBL are the chromosome translocation involving the c-*myc* proto-oncogene on chromosome 8, and the association with EBV. The evidence linking EBV to endemic BL is considerable. The virus was originally isolated from a cell line established from a BL biopsy (Epstein *et al.*, 1964), which led to intense speculation as to its role in the aetiology of the disease. Geser and colleagues detected EBV DNA in 51 out of 53 (96%) confirmed cases of BL in Uganda (Geser *et al.*, 1983), indicating a close association between the endemic form of BL and EBV. Seroepidemiological evidence for an aetiological link between eBL and EBV was provided in a prospective study of 42000 children in Uganda (de The, *et al.*, 1978). Of the 42000 chidren examined 12 developed tumours confirmed as, or histologically consistent with, BL during the 5 year study period. Of these, 10 had antibody titres to the EB viral capsid antigen (VCA) 8-10 fold higher than matched controls. These elevated antibody titres were detectable 18-24 months prior to clinical onset of BL. The authors concluded that these data '..supported a causal relationship between the Epstein-Barr virus and Burkitt's lymphoma..'.

Circumstantial evidence for the role of EBV as a virus of oncogenic potential is provided by its ability, unique amongst the human herpesviruses, of infecting and immortalising human peripheral B cells, *in vitro*, to form lymphoblastoid

cell lines (LCL) (Pope *et al.*, 1968). The formation and characteristics of these cell lines will be discussed in detail later in this chapter (section 5.3), however it should be noted that there are several phenotypic differences between the *in vitro* established lymphoblastoid cell and the Burkitt's lymphoma cell. In addition, the LCLs are not considered truly transformed cells, in that unlike BL cell lines, they do not generally grow in soft agar or produce tumours when inoculated sub-cutaneously into nude mice (Nilsson *et al.*, 1977). Further circumstantial evidence for a possible oncogenic role for EBV is its ability to induce tumours when inoculated into cotton-top tamarins (Shope *et al.*, 1973; Epstein *et al.*, 1985). These tumours, however, tend to be phenotypically more lymphoblastoid than BL-like (Young *et al.*, 1989b; Allday *et al.*, 1990).

Balanced against the wealth of data supporting a role for EBV in the aetiology of eBL is the fact that not all cases of BL are associated with EBV. Outside of areas where BL is endemic the association with EBV is much less marked, with only 15% of sBL cases being characterised by the presence of EBV (Lenoir, 1986). It can, of course, be argued that the sporadic and endemic forms of BL are completely separate entities; occurring, as they appear to, in a different stages of B cell differentiation. However, even within areas where BL is endemic, up to 4% of cases appear not to be associated with EBV. This is despite the fact that these patients are infected with EBV, as determined by serological methods (Geser *et al.*, 1983). Also, over 90% of the adult population is infected with EBV (Henle and Henle 1979a), yet outside of endemic areas BL is extremely rare, and when associated with EBV even rarer. Thus whilst EBV may be aetiologically associated with eBL it is clear that other co-factors are probably involved (section 4.9).

4.8 The BL cell phenotype

Cells taken from eBL biopsy samples express B cell antigens characteristic of a germinal centre B cell. They express both CD10 and CD77, but none of the B cell 'activation' markers, such as CD23, CD30 or CDw70 (Rooney *et al.*, 1986; Rowe M., *et al.*, 1987b; Ling *et al.*, 1989). In addition, BL cells express low, or absent, levels of the cell adhesion molecules LFA-1, LFA-3 and ICAM-1. (Clayberger *et al.* 1987, Pattaroyo *et al.*, 1988; Gregory *et al.*, 1988b).

A normal subset of B cells with a phenotype similar to BL cells has been isolated from germinal centre tonsillar B cells (Gregory *et al.*, 1987). These cells were identified as CD10+, CD77+, CD23-, CD30-, CDw70- B cells. They were

localised to the germinal centres of secondary lymphoid tissue, had the morphology of centroblasts and were actively cycling (Gregory *et al.*, 1987; Gregory *et al.*, 1988a). Thus a normal counterpart of BL cells has been identified, suggesting that the germinal centre may be the site of origin of BL cells, as had been suggested previously on histological grounds (Lennert and Mohri, 1978). It should be noted, however, that the tumour is only rarely found in lymph nodes (section 4.2).

Early passage, *in vitro* cultured, BL cells have a regular spherical morphology and grow a single cell suspension (Nilsson 1979; Rooney *et al.*, 1986; Rowe, M., *et al.*, 1987b). If BL cells are cultured for long periods of time, however, then phenotypic changes are observed in some, but not all, cell lines (Rooney *et al.*, 1986; Rowe, M., *et al.*, 1987b). Cells show increased homotypic aggregation, growing in large clumps of cells in a manner similar to *in vitro* established EBV immortalised lymphoblastoid cell lines (LCL) (discussed in section 5.3). In addition to the altered growth characteristics, changes in cell surface molecule expression are observed. Expression of the 'B cell activation markers' (CD23, CD30, CDw70) is observed coupled with a loss in expression of the early B cell makers (CD10 and CD77). BL cell lines have been categorised into four groups according to their phenotype, with group IV cell lines most resembling lymphoblastoid cells and group I cell lines resembling cells from BL biopsies (Rooney *et al.*, 1986; Rowe, M., *et al.*, 1987b). With respect to expression of the cell adhesion molecules (section 3.3), group I BL cells resemble biopsy cells in that they express low or absent levels of LFA-1, LFA-3 and ICAM-1 whereas group III cell lines express high levels of these molecules, similar to LCLs (Gregory *et al.*, 1987).

In addition to changes in surface phenotype and growth characteristics, BL cells which undergo phenotypic drift change with respect to viral gene expression. In group I BL cells a highly restricted pattern of EBV gene expression is observed with only one of the nuclear antigens (Epstein-Barr virus nuclear antigen [EBNA]-1, section 5.4) being expressed. In group III cell lines the full range of 'latency-associated' viral antigens are expressed (EBNA 1-6 and the latent membrane protein [LMP]), similar to the *in vitro* established lymphoblastoid cell lines (Rowe, M., *et al.*, 1987b; section 5.3). In addition to EBNA-1, BL cells also express two abundant small transcripts known as the EBERs (Rymo, 1979; Rowe, M., *et al.*, 1987b). These transcripts are untranslated and their function is not known.

References to BL phenotype in this study refer to the phenotype of group I, biopsy-like, cell lines, unless stated otherwise.

4.9 Malaria as a co-factor in eBL

Ever since Denis Burkitt described the geographical and climatic limitations of eBL (Burkitt, 1962) there has been speculation that some other environmental factor was involved in the aetiology of eBL. The most likely enviromental factor is holoendemic malaria, particularly that resulting from infection with *Plasmodium falciparum* (Kafuko and Burkitt, 1970; Morrow, 1985). There is a strong correlation between high incidence of BL and regions where malaria is holoendemic (Morrow, 1985). BL tends to be less common in urban areas, where the incidence of malaria is also lower (Biggar and Nkrumah, 1979), and BL is rare in patients with sickle cell anaemia, who are mostly resistant to malaria (Nkrumah and Perkins, 1976). In addition there is some, albeit inconclusive, data that reducing prevalence of malaria with anti-malarial drugs leads to a decrease in incidence of eBL (Geser and Brubaker, 1985).

4.10 Models for eBL development

There are two classic models proposed for the development of eBL. Both the model of Klein (1979a, 1987b) and that of Lenoir and Bornkamm (1987) envisage a multi-step scenario consisting of three stages. In Klein's model (1979a, 1987b) the first stage is *in vivo* immortalisation of B cells, following primary infection with EBV, as a result of which B cells are frozen at a particular stage of differentiation. The survival and proliferation of these cells would be facilitated by the immunosuppressive effects of continual malarial parasitaemia. The increased number of cell divisions would increase the probability of the third stage, a chromosomal translocation in one of the EBV infected B cells occurring as a random genetic event. This translocation would lead to activation of c-*myc* and to autonomous growth of a malignant B cell clone.

Lenoir and Bornkamm's (1987) model includes the same three genetic and environmental events, but places them in a different order. The first stage involves polyclonal B cell activation by chronic infection with malarial parasites. Secondly, a chromosome translocation involving the c-*myc* and Ig gene loci might occur in one or more pre-B cells undergoing V-D-J rearrangement. This translocation, and subsequent constitutive activation of the c-*myc* gene, would render the cells competent and responsive to growth

factors, but not transformed. These cells would eventually die unless the third vital event, infection with EBV, occurred. This, they propose, would render the cells independent of exogenous growth factors and allow outgrowth of a malignant B cell clone. In EBV-negative BLs they propose that infection with EBV is replaced by activation of a second co-operating oncogene.

For Lenoir and Bornkamm (1987) the experience of lymphoproliferative disease in AIDS patients was a strong argument against Klein's (1979a) hypothesis that malaria-induced T cell dysfunction favoured the outgrowth of EBV-infected B cells, in one of which a chromosome translocation subsequently occurred. AIDS patients with profound T cell dysfunction do develop EBV-positive B cell lymphoproliferative disease, but translocations are not generally observed, and BL is rare in this group of patients. In contrast patients with early stage AIDS, with a relatively intact T cell immune response have a relatively high incidence of BL (Kalter *et al.*, 1985; section 4.4). They considered the important stage in development of BL in AIDS patients to be continual antigenic stimulation, as a consequence of multiple infections, resulting in polyclonal B cell activation and lymphoid hyperplasia increasing the risk of a translocation occurring. In support of this hypothesis, there is an isolated report of B cell clones with t(8;14) translocations isolated from the lymph nodes of AIDS patients with generalised lymphadenopathy (Alonso *et al.*, 1987).

There are criticisms of both models (Lenoir and Bornkamm, 1987; Klein, 1987b), but it seems likely that, irrespective of order, the three stages identified in both models are critical to the development of eBL. A major criticism of the Lenoir and Bornkamm (1987) scenario was that they proposed the translocation in endemic BL occurred at the pre-B cell stage yet the tumour is predominantly one of resting mature B cells (section 4.8). Since EBV had been shown to infect pre-B cells why were more BLs with a pre-B cell phenotype not seen (Klein, 1987b). Klein's criticism, however, assumes that the translocation is the event which leads to activation of c-*myc*. If, as became apparent, a critical event in the activation of c-*myc* in eBL is the generation of point mutations and deletions within c-*myc* regulatory regions (section 4.5), then the translocation could be viewed as a preliminary stage in activation of c-*myc*. Thus, an ammended version of Lenoir and Bornkamm's (1987) model proposes a fourth stage involving mutations within c-*myc* regulatory regions occurring during the somatic mutation of rearranged V region genes in the germinal centre centroblasts, resulting in constitutive activation of the gene (Ling *et al.*, 1989). This theory would be consistent with the identification of germinal centre

centroblasts as a normal cellular counterpart of BL cells (Gregory *et al.*, 1987, 1988a). In addition, one cannot rule out the possibility of translocational activation of c-*myc* occurring during V-D-J rearrangement, but not becoming pathologically apparent until c-*myc* would normally be down-regulated (Gregory *et al.*, 1987).

4.11 Involvement of other cellular oncogenes

In eBL it is possible that EBV may co-operate with c-*myc* to cause malignant transformation, but there is, as yet, no evidence for any EBV-encoded gene functioning in such a manner. The two latency-associated proteins with putative oncogenic potential, EBNA-2 and LMP (section 5.4) are not expressed in BL cells (Rowe, D., *et al.*, 1986; Rowe, M., *et al.*, 1987b). Karran *et al* (1990) have reported that a sub-fragment of the viral genome spanning BamH1 A and I regions has immortalizing activity in primate epithelial cells, but similar activity in B cells has not been reported. Similarly, Wei and Ooka (1989) demonstrated that the gene product of the BARF-1 ORF, which is expressed during the virus lytic cycle, could transform rodent fibroblasts *in vitro*. It is not known whether expression of this gene in B cells has similar effects.

In sBL, gene transfer experiments transfecting NIH 3T3 cells with DNA from BL cells has resulted in the detection of activated ras genes Murray *et al.*, 1983; Lenoir, 1986). However these are isolated reports and widespread involvement of *ras* has not been demonstrated.

In further gene transfer experiments Diamond *et al* (1983) described activation of the human homologue (B-*lym*-1) of the chicken ChB*lym*-1 transforming oncogene in six BL cell lines of both sporadic and endemic type. The authors suggested that both B-*lym* and c-*myc* might be involved in the multi-step genesis of BL. However, the homology of the putative human B-*lym*-1 gene to the chicken ChB*lym*-1 gene, and the transforming ability of B-*lym*-1 has been challenged (Rogers, 1986; Devine, 1986). Little other evidence has emerged on the role of this oncogene in development of BL.

5. EPSTEIN-BARR VIRUS

5.1 The virus

Epstein-Barr virus (EBV) is a ubiquitous member of the human herpesvirus family. It has a worldwide distribution with over 90% of the adult population showing evidence of past infection (Henle and Henle, 1979a). The virus was first identified by Epstein and colleagues who observed viral particles morphologically similar to herpesviruses in cells cultured from an African BL biopsy (Epstein *et al.*, 1964). EBV is an enveloped virus containing a linear double-stranded DNA molecule (172kb) contained within an icosahedral nucleocapsid (Epstein and Achong, 1979). The viral genome is composed of tandemly arranged internal repeat sequences (IR) of 3072bp and a single long (UL) and short (US) unique sequence. A terminal region (TR) of 4-12 500bp repeat sequences is thought to be involved in formation of covalently closed DNA circles (episomes) following entry of the virus into the cell (Lindahl, *et al.*, 1976). The complete genome of virus isolated from the B95-8 cell line has been sequenced (Baer *et al.*, 1984). The restriction fragment map obtained with the BamH1 restriction enzyme, complete with the regions known to code for EBV proteins, is indicated in figure 4. There are over 90 predicted ORFs in the EBV genome. They are designated by a four letter and number acronym; for example, BZLF1 refers to the first leftward ORF of the BamH1 Z region of the genome (Baer *et al.*, 1984).

5.2 Viral tropism and persistence

EBV exhibits a dual cell tropism *in vivo* infecting both epithelial and B cells. Infection of B cells *in vivo* has long been established. EBV-positive B cells can be detected in the peripheral blood of patients with infectious mononucleosis (IM) (Klein *et al.*, 1976; section 5.7.1), and spontaneously arising EBV-positive immortalised B cell lines can also be established from the peripheral blood of EBV seropositive individuals (Nilsson *et al.*, 1971). Recovery of EBV from the saliva of patients with IM and normal healthy EBV seropositive individuals indicated an epithelial cell tropism (Golden *et al.*, 1973; Yao *et al.*, 1985). Replication of the virus in epithelial cells of the oropharynx has clearly been demonstrated (Lemon *et al.*, 1983; Sixbey *et al.*, 1984), and infection of epithelial cells of the uterine cervix has also been described (Sixbey *et al.*, 1986).

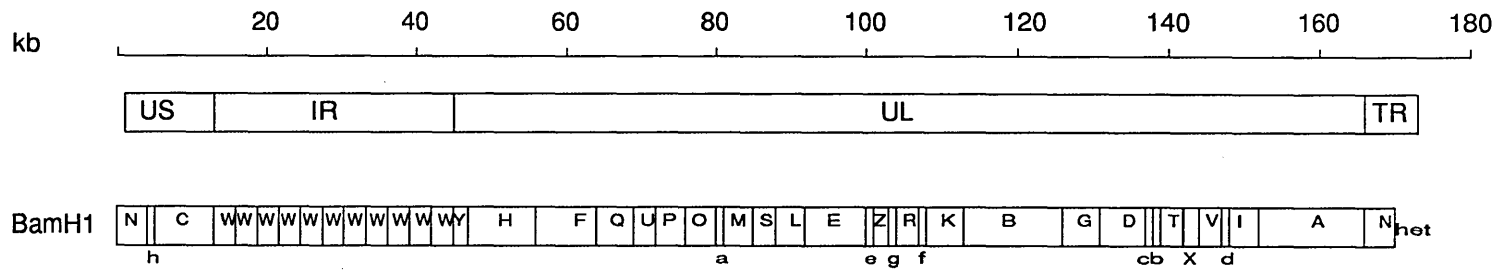
Infection of B cells *in vitro* is known to occur via CD21 (CR2), whose normal role is to act as a receptor for the C3d component of complement (Fingeroth *et al.*,

Legend: Figure 4

BamH1 restriction endonuclease map of the Epstein-Barr virus genome

Diagram showing the organisation of the Epstein-Barr viral genome. The BamH1 restriction endonuclease map of the B95-8 strain of virus is also shown. The scale is in kilo-base pairs (kb). US - unique short region; IR - internal repeat region; UL - unique long region; TR - terminal repeat region.

Figure 4


kb
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BamH1
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1984; section 3.2). It is assumed that infection of B cells *in vivo* occurs via the same route. EBV has been shown to bind to a CD21-like molecule on epithelial cells and infection is thought to occur via this molecule (Young *et al.*, 1986; Sixbey *et al.*, 1987; Young *et al.*, 1989c).

In common with other herpesviruses, EBV establishes a persistent infection following primary infection. The site of viral persistence is not firmly established and is the subject of some debate. The two proposed sites for EBV persistence are B cells and epithelial cells. Initially, before the virus tropism for epithelial cells was established, it was widely believed that the virus must persist in B cells since these were the only cells which could be infected with EBV, and spontaneously arising EBV-infected B cell lines could be established by *in vitro* culture of peripheral blood from infected individuals (Klein 1989). The discovery of an autologous cytotoxic T-lymphocyte response to EBV infected B cells and the demonstration that infection of B cells *in vitro* led to rapid expression of molecules recognised by EBV-specific cytotoxic T-lymphocytes (CTL) (Moss *et al.*, 1978, 1981) led to the hypothesis that the site of persistence was the epithelial cell compartment (Moss *et al.*, 1981). It was proposed that infection of B cells occurred as the cells passed through the region of the oropharynx and infected B cells were continuously eliminated by EBV-specific CTL. This theory was strengthened by the discovery of virus replication in epithelial cells (Lemon *et al.*, 1983; Sixbey *et al.*, 1984). More recently, it has been proposed that the site of persistence within the epithelial cell compartment is the basal stem cell layer and virus production is linked to differentiation with virus being released from the terminally differentiated squamous cells (Sixbey *et al.*, 1987; Allday and Crawford 1988). This hypothesis is consistent with the observation that, in *in vitro* cultures, only the EBV nuclear antigen complex (section 5.4) is detected in immature epithelial cells and lytic cycle antigens are only detectable in the more mature cells (Sixbey *et al.*, 1983, 1984). More recently this hypothesis has been challenged by the observation that two patients undergoing bone marrow transplantation 'lost' the pre-transplant strain of virus. In one case the patient acquired the virus of the bone marrow donor and the other patient became reinfected at a later date with another strain of virus (Gratama *et al.*, 1988). The implication of these results was that the compartment in which the virus was persisting had been destroyed by the pre-transplant irradiation and chemotherapy, and that the most likely compartment was cells of the haemopoietic system which were eliminated by the treatment. Whilst these results appear to favour the B cell hypothesis the data was from two patients only, and considerable damage to

epithelial cells can occur during such treatment. In support of the B cell hypotheisis is the findings of Yao *et al* (1989) who demonstrated that EBV-infected B cells are long-lived *in vivo* and thus presumably invisible to EBV-specific CTL. A major problem with the B cell theory is explaining how virus is spread. *In vitro* EBV-immortalised B cells are generally poor producers of virus, and cell death occurs as a consequence of virus production (Zur Hausen *et al.*, 1967). Furthermore, viraemia has not been demonstrated *in vivo* (Rickinson *et al.*, 1975), and there have been no reports of VCA positive circulating B-cells. Whilst this does not rule out the possiblility of B cells being the site of EBV persistance it does indicate that the main site of viral replication and production is likely to be the epithelial cell compartment.

5.3 *In vitro* immortalisation of B cells

Unique amongst the human herpesviruses is EBV's immortalising property. *In vitro* infection of human peripheral blood with EBV results in the formation of immortalised B cell lymphoblastoid cell lines (LCL) (Pope *et al.*, 1968). LCLs have been generated by infection of a variety of B cell sub-populations including pre-B cells (Kubagawa *et al.*, 1988) and germinal centre B cells (Gregory *et al.*, 1988a). Entry of the virus into the B cell via CD21 is followed by viral genome circularization to form an episome, a step thought to be essential for immortalisation (Hurley and Thorley-Lawson, 1988). Seven to fourteen days after infection the viral episome is amplified (Hurley and Thorley-Lawson, 1988), and the virus is maintained in the episomal form with integration into host chromosomes thought to be a rare event (Nonoyama and Pagano, 1972). Infection of B cells *in vitro* is characterised by the expression of a characteristic pattern of latency-associated antigens (section 5.4). *In vitro* experiments, infecting prolymphocytic leukaemic B cells with EBV, revealed an ordered sequential pattern of latency associated gene expression. Approximately eight hours after infection expression of one of the nuclear antigens (EBNA-2) is observed. Expression of the other nuclear antigens (EBNA-1, EBNAs 3-6) is observed twelve to eighteen hours after infection. Finally the EBV latent membrane protein (LMP) is expressed after 40 hours (Allday *et al.*, 1989). This sequence of events has been confirmed following infection of normal B cells (M. Doyle, unpublished data). The great majority of cells within an established LCL are 'latently' infected, expressing only the latency associated antigens (EBNAs 1-6 and LMP) (Rowe *et al.*, 1986; Rowe *et al.*, 1987; Ricksten *et al.*, 1988; Allday *et al.*, 1988). In most LCL, however, a small proportion of cells are lytically infected; that is the viral lytic cycle is activated, viral lytic cycle antigens

expressed (section 5.5) and infectious virus produced and released from the cell. Entry into the lytic cycle is incompatible with continued cell proliferation and it is thought that lytically infected cells, *in vitro*, are those which are undergoing terminal differentiation (Crawford and Ando, 1986). The proportion of cells within an LCL in the lytic cycle appears dependent on the origin of cells infected with EBV. LCLs derived by infecting lymphocytes from the peripheral blood of EBV seronegative adults generally have a higher proportion of cells expressing lytic cycle antigens than LCLs derived by infecting cord blood lymphocytes (Crawford *et al.*, 1979). The proportion of cells in the lytic cycle can be increased by a variety of means including treatment with phorbol esters and superinfection with the P3HR-1 strain of EBV (Zur Hausen *et al.*, 1978; Rabson *et al.*, 1983). P3HR-1 is an unusual cell line in that even though a relatively large proportion of cells are in the lytic cycle at any one time (1-5%) the virus produced is non-immortalising (Rabson *et al.*, 1982). This lack of immortalising activity is due to a deletion within the viral genome encompassing the EBNA-2 gene (Jeang and Hayward, 1983; section 5.4). Rearranged EBV DNA from this strain of virus has permitted the identification of a viral gene which controls the switch from latency to virus production (BZLF-1, section 5.5).

Lymphoblastoid cells have an irregular morphology and grow in large clumps of cells (Nilsson 1979). They are considered non-transformed cell lines, in that they generally do not grow in soft agar or produce tumours when inoculated subcutaneously into nude mice (Nilsson *et al.*, 1977).

EBV-immortalised lymphoblastoid cells have an activated phenotype expressing CD20, CD21, CD23, CD30, CDw70 and CD71, in addition to the pan B cell markers CD19 and CD22 (Rowe, M., *et al.*, 1985; Ling *et al.*, 1987; Stein *et al.*, 1989; Schwarting and Stein, 1989; Ling *et al.*, 1989). CD10 and CD77 are not highly expressed in these cell lines. LCLs also express high levels of the cell adhesion molecules LFA-1, LFA-3 and ICAM-1 (Patarroyo *et al.*, 1988; Gregory *et al.*, 1988b) (section 3.3). The homotypic cell aggregation of LCLs is known to be mediated, at least in part, by interaction between LFA-1 and one of its ligands ICAM-1 (Rothlein *et al.*, 1986).

5.4 Latency associated antigens

There are nine known latency associated antigens; six nuclear antigens (EBNA 1-6), the latent membrane protein (LMP) and two terminal proteins (TP1 and TP2). There is some considerable confusion over nomenclature. Whilst the

names EBNA-1 and EBNA-2 are generally accepted, EBNAs 3, 4, and 6 (Ricksten *et al.*, 1988; Allday *et al.*, 1988) are also known as EBNAs 3a, 3b and 3c respectively (Petti and Kieff, 1988, Petti *et al.*, 1988). EBNA-5 (Dillner *et al.*, 1986) is also known as EBNA-leader protein (LP) (Sample *et al.*, 1986) or, more confusingly, EBNA-4 (Rowe D, *et al.*, 1987). There is no dispute over LMP, but TP1 and TP2 (Laux *et al.*, 1988; 1989) have also been termed LMP2A and LMP2B respectively (Sample *et al.*, 1989). This study will use the nomenclature EBNAs 1-6, LMP, TP1 and TP2.

EBNA-1 encoded by a gene within the BamH1 K region of the genome, varies in size from 65 - 85kDa depending on the viral strain (Strnad *et al.*, 1981). Functionally, it is known to be required for maintenance of the episomal form of the viral DNA, and binds to the EBV plasmid origin of replication (OriP) *in vitro* (Yates *et al.*, 1984; Yates *et al.*, 1985). EBNA-1 has also been shown to transactivate an enhancer, within the OriP region of the genome, which is active in immortalised cells (Sugden and Warren 1989). In addition to participating in DNA replication EBNA-1 is apparently involved in positively regulating its own synthesis (Sugden and Warren, 1989). It is not known whether EBNA-1 can transactivate host cell promoters, but it is not inconceivable that EBNA-1 might act to promote host cell DNA replication by binding to homologous sequences in host cell DNA (Knutson and Sugden, 1989).

Two distinct EBNA-2 alleles (A and B), isolated from different strains of virus, have been described. A-type viral isolates, including the B95-8 strain, encode a 85kDa protein (EBNA-2A) and type-B isolates (such as AG876) encode a shorter (75kDa) protein (EBNA-2B) (Dambaugh *et al.*, 1984). Both proteins are encoded from the BamH1 W, Y and H regions of the genome. It was originally believed that type B viral strains were found mainly in areas where BL was endemic whereas type A viruses were found worldwide (Zimber *et al.*, 1986). More recent data, however, has indicated that B-type viral strains are more widely disseminated than was first believed. Sixbey *et al* (1989) detected EBV in the throat washings of 34 out of 157 healthy North American individuals. Of those 41% were infected solely with B-type virus, 50% with A-type virus and 9% with both types. Dambaugh *et al* (1984) suggested that biological differences between the two types of virus might exist; and Rickinson and colleagues (1987) described phenotypic differences between LCLs immortalised with the the two strains, including lower growth rates and saturation densities in B-type LCLs when compared to A-type LCLs.

EBNA-2A is known to be required for *in vitro* immortalization of B cells. Initial evidence for its role in immortalization was provided by the identification of a non-immortalizing viral strain (P3HR-1) which lacked EBNA-2 coding sequences (Rabson *et al.*, 1982). Replacement of this gene by recombination and complementation restores immortalizing function (Hammerschmidt and Sugden 1989; Cohen *et al.*, 1989). Transfection of EBV-negative BL cell lines with EBNA-2A induces expression of the B cell surface molecules CD21 and CD23. The induction of CD23 expression, but not CD21, by EBNA-2A was enhanced in cells co-transfected with LMP (Wang F., *et al.*, 1987; Wang F., *et al.*, 1990a). Transfection with EBNA-2B does not induce similar phenotypic changes (Wang F., *et al.*, 1990a). It has also recently been demonstrated that both EBNA-2A and EBNA-2B can induce expression of LMP (Abbott *et al.*, 1990; Wang F., *et al.*, 1990b). The mechanism of induction is transcriptional, possibly by interaction with an EBNA-2 response element located upstream of the LMP gene (Wang F., *et al.*, 1990b). In similar experiments, however, Cordier *et al* (1990) found that whilst EBNA-2A induced expression of CD21 and CD23, it did not induce expression of LMP. Azim *et al* (1990) also reported the presence of EBNA-2 positive, LMP negative, CD23 negative B cells following infection of peripheral blood mononuclear cells by EBV. The reasons for the different findings are not clear.

EBNA-5 has a wide range of reported molecular weights ranging from 41-70kDa, and a ladder of 6 EBNA-5 polypeptides is observed in cells recently infected with B95-8 virus (Dillner *et al.*, 1986). The EBNA-5 coding region is predominantly composed of a series of internal repeat sequences. It is thought that the heterogeneity in sizes observed is either a consequence of differing numbers of internal repeats per genome, use of different promoters or differential splicing (Dillner and Kallin, 1988; Finke *et al.*, 1987). The function of EBNA-5 is not clear. It has been shown that EBNA-5 is not required for *in vitro* immortalization of B cells, although it is possible that it may enhance growth of immortalized B cells (Hammerschmidt and Sugden, 1989).

Little is known of the function of EBNAs 3, 4 and 6, although EBNA-6 has been shown to induce expression of CD21 when transfected into EBV-negative BL cells (Wang F., *et al.*, 1990a).

There are three membrane proteins expressed in latent infection; namely LMP, TP1 and TP2. LMP is a 58-63kDa membrane protein encoded by the first leftward ORF of the BamH1 N_{het} region (BNLF-1) of the viral genome

(Fennewald *et al.*, 1984). As mentioned above there is some evidence for transactivation of LMP expression by EBNA-2 and, like EBNA-2, LMP has also been implicated in the *in vitro* immortalization process. When transfected into Rat-1 and Balb 3T3 cells the gene appears to have transforming activity, resulting in anchorage independent growth of transfected cells (Wang, D., *et al.*, 1985; Baichwal and Sugden, 1988). Recently, transfection of LMP into an immortalised human epithelial cell line was reported as inducing a morphologically transformed phenotype (Fahraeus *et al.*, 1990). It is not known how LMP achieves transformation in these cells but it has structural similarities to ion channels and growth factor receptors (Sugden, 1989). In addition to receptor-like structure the molecule also has a very rapid turnover rate (2 - 5 hours), a common characteristic of growth factor receptors (Baichwal and Sugden, 1987; Mann and Thorley-Lawson, 1987). Transfection of an EBV-negative BL cell line with LMP results in increased expression of the cell adhesion molecules, LFA-1, LFA-3 and ICAM-1 (Wang D., *et al.*, 1988b) (section 3.3). It has been proposed that LMP might also affect the function of these molecules. Expression of LMP in B-JAB, an EBV negative B cell line, which expresses high levels of LFA-1 and ICAM-1, results in increased homotypic cell adhesion, with no additional increase in expression of the two molecules, suggesting that LMP might induce higher affinity binding (Wang F., *et al.*, 1990a). Increased expression of the B cell activation markers CD23 and CD71 (section 3.2) and an increase in intracellular free calcium was also observed (Wang D., *et al.*, 1988b), suggesting a role for LMP in EBV-induced proliferation of B cells. A truncated form of LMP, expressed in lytically infected cells, has also been identified (Modrow and Wolf, 1986). This protein lacks 128 amino acids from the N-terminus of the protein and, when transfected into EBV-negative BL cells, has none of the phenotypic effects of the full length protein (Wang D., *et al.*, 1988b). The truncated form of LMP is also non-transforming when transfected into rodent fibroblasts (Wang, D., *et al.*, 1988a; Baichwal and Sugden, 1988). Liebowitz *et al* (1987) described an association between LMP and vimentin intermediate filaments which was not detected with the truncated form of the protein (Wang D., *et al.*, 1988a) prompting the authors to speculate that activity of LMP might require interaction with vimentin. This seems unlikely since, in some cell lines at least, LMP can function independently of vimentin (Liebowitz and Kieff, 1989). Interestingly, limited sequence and structural similarities between vimentin and *fos*, *jun*, CREB and *tpr* transcription factors have recently been described, suggesting a potential role for vimentin in signal transduction between the cell membrane and nucleus (Capetanaki *et al.*, 1990).

In epithelial cells transfection of LMP results in inhibition of terminal differentiation, suggesting a potential role for LMP in the aetiology of nasopharyngeal carcinoma (NPC) (Dawson *et al.*, 1990). NPC is a tumour of poorly differentiated epithelial cells associated with EBV, and in which only EBNA-1 and LMP are expressed (Klein *et al.*, 1979b; Fahraeus *et al.*, 1988; Young *et al.*, 1988).

Hudson *et al* (1985) described two transcripts (2.0 and 1.7kb) actively expressed from the unique short region of the B95-8 genome. These transcripts were later shown to be derived from a coding sequence which spanned the two termini of the genome (BamH1 N_{het} and BamH1 C), and thus could only be expressed from circularized episomal, or tandemly arranged integrated, viral DNA (Laux *et al.*, 1988; Laux *et al.*, 1989; Sample *et al.*, 1989). The two putative proteins were designated TP1 and TP2 (from the 2.0 and 1.7kb transcripts respectively). *In vitro* translation demonstrated proteins of 53kDa (TP1) and 40kDa (TP2) (Sample *et al.*, 1989). Both were predicted to be membrane proteins with multiple transmembrane domains. Recent data has indicated that TP1 co-localises, in part, with LMP and antibodies to TP1 have demonstrated expression of the protein in both LCL and BL cell lines (Longnecker and Kieff, 1990; Rowe D, *et al.*, 1990). Less is known about the expression of TP2. The function of the TP proteins is unclear, but since circularization of the viral genome is apparently required for immortalisation (Hurley and Thorley-Lawson 1988), it is tempting to speculate that the protein is involved in the *in vitro* immortalisation process. Recently Frech *et al* (1990) have used an anti-TP1 polyclonal serum to detect TP1 in both LCL and BL cell lines, although the phenotype of the BL cell lines was not indicated.

5.5 The lytic cycle

In contrast to the latent state, a large number of genes are active during the lytic cycle. Entry into the lytic cycle, *in vitro*, occurs when the the BZLF1 ORF is expressed (Countryman and Miller, 1985; Grogan *et al.*, 1987). BZLF-1 was identified using the P3HR-1 cell line in which a higher than usual proportion of cells are in the lytic cycle. The gene encoded by BZLF-1 is usually inactive in latently infected cells, but in the P3HR-1 cell line rearranged heterogenous EBV DNA (het DNA) was isolated in which juxtaposition of BZLF-1 and a promoter active in latent cells has occurred (Countryman and Miller, 1985). Transfection experiments have clearly demonstrated that the gene product encoded by BZLF-1 is capable of inducing the switch from latency to virus production (Grogan *et*

al., 1987). The gene product of BZLF1 acts as a transactivator of immediate early lytic cycle genes (Rooney *et al.*, 1989) and is thought to initiate a cascade of sequential expression of immediate early, early and final late gene products. It is not known what activates expression of BZLF1, but recently TPA response elements have been identified upstream of BZLF-1, suggesting that TPA induces the lytic cycle by interacting with these elements (Flemington and Speck, 1990a). The same authors also demonstrated that the *fos/jun* transcription factor complex could bind to this site. The BZLF-1 gene product has homology to c-*fos* and can bind to the AP-1 transcription factor binding site (Farrell *et al.*, 1989). BZLF-1 has also been shown to positively regulate its own transcription (Flemington and Speck, 1990b).

Three immunogenic lytic cycle antigen complexes have been identified on the basis of their ability to react with human anti-sera. They are the membrane antigen (MA), early antigen (EA) and the viral capsid antigen (VCA) complexes (Pearson and Luka, 1986).

There are a large number of proteins which comprise the EA complex, of which three are immunogenic. Human EBV-immune sera recognise polypeptides of 85 and 140kDa and a family of polypeptides of 48-55kDa (Pearson and Luka, 1986). Two forms of EA are observed by indirect immunofluorescence using human sera, termed diffuse (EA-D) and restricted (EA-R). EA-D is found in the cytoplasm and nucleus of infected cells whereas EA-R is restricted to the cytoplasm (Henle G., *et al.*, 1971). The 48 - 55kDa polypeptides comprise the EA-D component, whereas the 85kDa polypeptide is the restricted form (Luka *et al.*, 1986). The 140kDa protein appears not to be a part of either EA-D or EA-R. The early gene products are assumed to be involved in synthesis of viral DNA, although the precise function of the majority of early proteins remain unclear (Dillner and Kallin, 1988).

VCA, as its name implies, forms the structural components of the virus capsid and is expressed late in the lytic cycle. There are at least three major polypeptides of 125, 152 and 160kDa (Pearson and Luka, 1986). The major structural component is the 160kDa protein (Vroman *et al.*, 1985). Antibodies to the VCA complex are a frequently used indicator of infection with EBV, and the major immunogenic components of VCA are thought to be the 125kDa and 160kDa proteins (Luka *et al.*, 1984; Vroman *et al.*, 1985).

The membrane antigen complex is comprised of three glycoproteins - gp85,

gp220 and gp340 - located in the viral envelope and membrane of infected cells (reviewed in Dillner and Kallin 1986). The MA complex is thought to be involved in attachment and entry of the virus into the cell (Dillner and Kallin 1986). Gp340, is known to elicit neutralising antibodies (North *et al.*, 1982), and attempts to develop a vaccine for EBV have centred on this molecule (Epstein and Morgan, 1986).

5.6 Immunological control of the virus

The control of persistent EBV infection is complex, involving both humoral and cell mediated immune (CMI) responses (Rickinson, 1986). The importance of T cell mediated immunity to EBV is illustrated by patients receiving immunosuppressive therapy. In these patients EBV-associated lymphoproliferative disease is a relatively common event (Thomas *et al.*, 1990a; section 5.7.2). The demonstration of an impaired CMI response in these patients (Crawford *et al.*, 1981b) suggested that the T cell immune response was of fundamental importance in controlling proliferation of EBV-infected B cells.

In normal individuals, following primary infection and establishment of a persistent carrier state, unchecked proliferation of virus-infected B cells is thought to be prevented by EBV-specific cytotoxic T-cells (CTL). The presence of these cells was demonstated clearly by Moss *et al* (1978) using an *in vitro* assay and observing complete regression of proliferating EBV-immortalised B cells caused by T cells from the peripheral blood of normal EBV-seropositive individuals. These T cells are classical CD8 positive, HLA class I restricted CTL (Wallace *et al.*, 1982).

A number of the viral latency-associated proteins have been identified as epitopes for EBV-specific CTL. As a membrane protein, LMP was considered a prime candidate for recognition by EBV-specific CTL, and such an interaction has been demonstrated (Thorley-Lawson and Israelsohn, 1987; Murray *et al.*, 1988a).

The demonstration by Townsend and co-workers (Townsend *et al.*, 1986) that peptides derived from the influenza nucleoprotein could be presented to T cells on the cell surface led to attempts to identify T cell epitopes amongst the EBNAs. EBNA-2 was proposed as an epitope for EBV-specific CTL on the basis that HLA-restricted CTL clones could be isolated which killed LCLs immortalised with A-type but not B-type virus (Moss *et al.*, 1988). However, the

subsequent demonstration that the differences between the two types of virus extended to EBNAs 3, 4 and 6 (Rowe M., *et al.*, 1989; Sample *et al.*, 1990; section 5.4) called into question the conclusions of Moss *et al* (1988), on the grounds that the other EBNAs might be acting as targets for CTL. Recent work, however, indicates that epitopes on EBNA-2A are indeed recognised by EBV-specific T cells (Murray *et al.*, 1990). In addition to EBNA-2, peptides derived from EBNA-3 and EBNA-6 have been described acting as epitopes for EBV-specific T cells (Burrows *et al.*, 1990a, 1990b; Murray *et al.*, 1990). It remains to be seen whether EBV-specific CTL epitopes are present in EBNA-1, EBNA-4 and EBNA-5.

The humoral response, by contrast, appears relatively unimportant in controlling persistent infection, as evidenced by the fact that immunosuppressed patients with EBV-associated lymphoproliferative disease have normal or elevated EBV-specific antibody titres (Henle W., and Henle G., 1981).

It has been reported that some BL cell lines are resistant to EBV-specific T cell lysis (Rooney *et al.*, 1985), and down-regulation of HLA class I, in particular HLA class A11 (Masucci *et al.*, 1987) has been suggested as a mechanism by which BL cells may evade virus specific T cell lysis. In addition, it has been suggested that evasion of T cell lysis is a consequence of the BL cells not expressing the EBV antigens known to act as targets for EBV-specific CTL. None of the EBV antigens known to be recognised by EBV-specific CTL (see above) are expressed in BL cells (Rowe, M., *et al.*, 1987b). It is not known whether the one nuclear protein expressed in BL cells (EBNA-1) can act as a target for EBV-specific CTL. Various workers have noted low or absent expression of cell adhesion molecules on BL cells and proposed that down-regulation of these molecules may contribute to evasion of immunosurveillance (Clayberger *et al.*, 1987; Patarroyo *et al.*, 1988; Gregory *et al.*, 1988b). These cell adhesion molecules (LFA-1, LFA-3 and ICAM-1; section 3.3) are known to be important in adhesion of CTL to infected B cells, an essential stage prior to lysis (reviewed by Springer *et al.*, 1987; section 3.3). Gregory and colleagues (1988b) correlated expression of cell adhesion molecules and susceptibility to specific T cell lysis, with the phenotype of BL cell lines (section 4.8). Biopsy-like group I BL cell lines generally expressed low or absent levels of cell adhesion molecules and did not form conjugates with T cells. In contrast, group III, LCL-like, cell lines expressed high levels of adhesion molecules and formed conjugates with CTL.

Malaria is thought to be one of the co-factors in the development of BL (section 4.9) and Klein proposed that the immunosuppressive effects of malaria may assist the expansion of a pool of EBV immortalised B cells, increasing the risk of a rare chromosomal translocation occurring (Klein 1979a, 1987b). There is some evidence that chronic malaria may have an immunosuppressive effect, and impaired EBV-specific CTL responses have been described in residents of malaria endemic regions compared to non-endemic regions (Moss *et al.*, 1983). It has also been demonstrated that African children with acute malaria have an impaired CTL response to EBV, but that during convalescence the CTL response returns to normal (Whittle *et al.*, 1984). Recently, an increased number of EBV infected B cells have been observed in the peripheral blood of children with acute malaria compared to control subjects (Lam *et al.*, manuscript submitted for publication). However, as Lenoir and Bornkamm (1987) pointed out in their alternative scenario for BL development (section 4.10), if the immunosuppressive effects of malaria allow EBV-immortalised B cell to escape T cell surveillance and proliferate why is EBV-associated lymphoproliferative disease such as that seen in other immunosuppressed patients not observed?

5.7 Disease association

For the majority of individuals infection with EBV is asymptomatic. However, EBV is known to be the cause of infectious mononucleosis (Niederman *et al.*, 1970) and is aetiologically associated with two human malignancies, BL (section 4) and nasopharyngeal carcinoma (Klein 1979b). EBV is also implicated in the development of a number of other diseases including; X-linked lymphoproliferative syndrome (reviewed by Weisenbergen and Purtilo, 1986; Thomas *et al.*, 1990a), lymphoproliferative disease in immunosuppressed individuals (section 5.7.2) and oral hairy leukoplakia (Greenspan *et al.*, 1985). Only the disease associations of direct relevance to this study will be discussed here.

5.7.1 Infectious mononucleosis (IM)

The majority of individuals are infected with EBV early in life when infection is usually asymptomatic (Henle G., and Henle W., 1970). If, however, infection is delayed to adolescence then approximately 50% of those infected develop IM (Niederman *et al.*, 1970). IM is usually a benign, self-limiting disease of

adolescence. Infection occurs via the oral route and viral replication and shedding from pharyngeal epithelial cells is observed (Golden *et al.*, 1973; Sixbey *et al.*, 1984). The disease is characterised by polyclonal B cell activation and the appearance of atypical CD8 positive lymphocytes in the circulation (Crawford *et al.*, 1981a). Signs and symptoms include; pyrexia, lymphadenopathy, pharyngitis, splenomegaly and fatigue (Crawford and Edwards, 1990). Occasionally complications of pneumonitis and hepatitis are observed and, very rarely, the disease may be fatal (Weisenburgen and Purtilo, 1986). The usual outcome, however, is complete recovery 1-6 months after onset of illness; although rare patients, presumably as a consequence of an impaired T cell response to EBV, fail to recover and develop chronic IM (Borysiewicz *et al.*, 1986).

The serological response during the acute phase of IM is characterised by the presence of IgG and IgM class antibodies to VCA and IgG to EA (Henle W., and Henle G., 1979b). Antibodies to EBNA-2, but not EBNA-1, are also detectable early in infection (Henle W., *et al.*, 1987). During convalescence IgG antibodies to EA and anti-VCA IgM disappear. Antibodies to EBNA-1 appear and, along with IgG to VCA, persist for life. IM is also characterised by the appearance of heterophile antibodies (HA). HA are antibodies, usually IgM, which cause agglutination of erythrocytes of species other than human. HA arise early in infection and can persist for up to 12 months (Evans *et al.*, 1975).

5.7.2 EBV-associated lymphoproliferative disease in immunosuppressed individuals

EBV-associated lymphoproliferative disease is relatively common in patients with severe T cell dysfunction. The two groups of individuals most at risk are: i) those undergoing immunosuppressive therapy following organ tranplantation and, ii) individuals infected with HIV.

The introduction of cyclosporin A - an inhibitor of T cell function - to prevent graft versus host disease in allograft recipients led to the increased incidence of malignancy, in particular non-Hodgkin's lymphoma (NHL), in these patients (reviewed by Cleary *et al*, 1986). The presence of EBV in such tumours and impaired EBV-specific CTL responses in patients receiving CSA was clearly demonstrated (Crawford *et al.*, 1980, 1981b). It is now recognised that there is a broad spectrum of EBV-associated B cell lymphoproliferative disease in these patients, ranging from polyclonal B cell proliferations to true malignant

monoclonal NHL, usually of the large cell lymphoma type (Cleary *et al.*, 1986; Thomas *et al.*, 1990a). Where the tumours arise at multiple sites each site usually represents a different clonal population of B cells (Cleary *et al.*, 1986). The current theory for development of these tumours and involvement of EBV envisages EBV-induced polyclonal B cell activation which, in the absence of a T cell response to EBV, results in polyclonal proliferations. At this stage the lesions are still sensitive to T cell control should immunosuppression be removed. Subsequent, probably genetic, events result in the outgrowth of one or more monoclonal malignant lesions (Thomas *et al.*, 1990a). There is evidence that primary EBV infection in post-transplant patients is a significant risk factor for developing EBV-associated lymphoproliferative disease since a significant proportion of patients with EBV-associated lymphoproliferative disease are EBV-seronegative prior to transplant (Ho *et al.*, 1985; Thomas *et al.*, 1990b). Phenotypically the tumour cells resemble the *in vitro* immortalised lymphoblastoid cells, in terms of expression of B cell activation markers and EBV latency-associated antigens (Young *et al.*, 1989a; Thomas *et al.*, 1990b).

As has been mentioned earlier (section 4.4) HIV-infected individuals with early stage AIDS have an increased incidence of BL. In addition to BL, HIV-infected patients, particularly those with advanced AIDS have a relatively high incidence of NHL, of diffuse large cell type (Kalter *et al.*, 1985). The presence of EBV has been detected in some of these tumours, but not the chromosome translocations characteristic of BL (Knowles *et al.*, 1989). As with the post-transplant tumours the phenotype is more typical of an LCL than a BL cell (Knowles *et al.*, 1989).

6. AIMS OF THE PROJECT

The aim of this project was to examine the role constitutive high level expression of c-*myc* plays in determining the phenotype of endemic BL. In order to achieve this it was proposed to introduce, by transfection, plasmids encoding a constitutively expressed c-*myc* gene into *in vitro* EBV immortalised B cells (LCLs). As has been described earlier in this chapter, and is summarised in Table 1, there are a number of phenotypic differences between the phenotype of the *in vivo* EBV-infected BL cell and the *in vitro* infected LCL. The phenotype of the *myc*-transfected LCL would be compared to that of a control LCL and that reported for BL cells.

Table 1

Comparison of Burkitt's lymphoma (BL) and lymphoblastoid cell lines (LCL).

	BL	LCL
c-*myc* translocation	+	-
Morphology	regular	irregular
Growth pattern	single cell	clumps
Growth in soft agar	+	-
Tumours in nude mice	+	-
EBNA-1	+	+
EBNA 2-6	-	+
LMP	-	+
TP	?	+
Cell adhesion molecules	-	+
HLA class I	+ or low	+
CD10, CD77	+	-
B cell activation markers	-	+

CHAPTER II

RESULTS I

CONSTITUTIVE EXPRESSION OF C-*MYC* IN HUMAN LYMPHOBLASTOID CELL LINES

1. INTRODUCTION

1.1 Experimental Design

In order to mimic the activation of c-*myc* seen in BL, plasmid constructions, in which the c-*myc* gene was constitutively expressed, were introduced into human LCLs. The same constructions also encoded a selectable marker allowing selection of transfected cells with the antibiotic hygromycin B. Once stable transfected cell lines were established the phenotypic characteristics of those cell lines could be compared with that of a control LCL transfected with a plasmid construction containing the hygromycin B resistance gene alone, and the reported phenotype of BL cell lines (Chapter I, section 4.8).

1.2 Plasmid DNA constructions

1.2.1 pSV3Neo (Southern and Berg, 1982)

pSV3neo (figure 5a) contains pBR322 sequences, including the pBR322 origin of replication and the B-lactamase gene which allows selection in permissive bacterial cells with the antibiotic ampicillin. Also contained on this plasmid are the *neo* and SV40 large T antigen (LT) genes under control of SV40 early region promoter/enhancer elements. *Neo* encodes a phosphotransferase which inactivates the antibiotic G418, allowing selection of transfected cells in mammalian cells (Southern and Berg 1982). Replication in mammalian cells is facilitated by the SV40 origin of DNA replication.

1.2.2 pHEBoSV (Lombardi *et al.*, 1987)

pHEBoSV (figure 5b) comprises the SV40 early region promoter/enhancer element inserted into the pHEBo plasmid (Sugden *et al.*, 1985). pHEBo contains the *E.Coli* gene, *hph*, which encodes a hygromycin B phosphotransferase. When expressed, this gene confers resistance to the antibiotic hygromycin B in mammalian cells (Sugden, *et al.*, 1985). pHEBo also contains the EBV origin of plasmid replication (OriP) which allows the plasmid to be maintained episomally within cells expressing EBNA-1 (Yates, *et al.*, 1984).

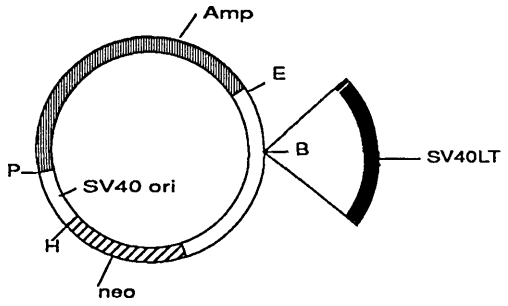
Legend: Figure 5

Schematic maps of plasmid DNA constructions used in transfection experiments.

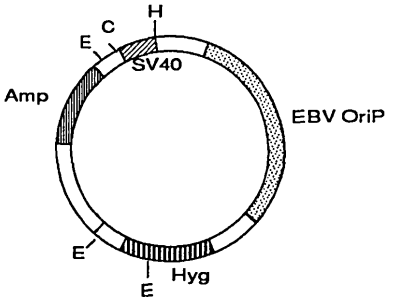
Diagram showing organisation of plasmid DNA constructions used in transfection experiments: a) pSV3Neo; b) pHEBoSV; c) pHEBoSVmyc2,3; d) pHEBoSVmyc1,2,3. Amp - β-lactamase gene of *E.coli* conferring ampicillin resistance; SV40LT - DNA from SV40 encoding SV40 large T antigen; SV40 ori - SV40 origin of replication; Neo - gene encoding resistance to the antibiotic G418; Ori P - EBV origin of episomal plasmid replication; Hyg - *hph* gene encoding hygromycin B phosphotransferase; SV40 - SV40 early region promoter/enhancer element; Ex - exon. Sites of the restriction enzymes AluI (A), BamHI (B), ClaI (C), EcoRI (E), HinDIII (H), PvuII (P) and XhoI (X) are indicated.

Figure 5

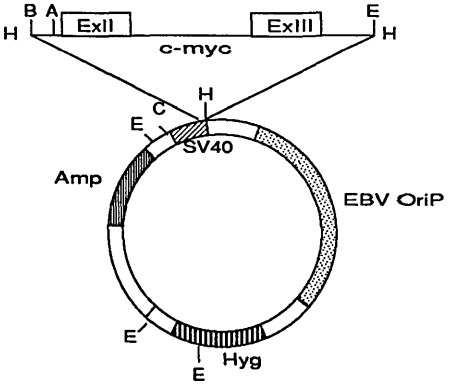
a) pSV3Neo



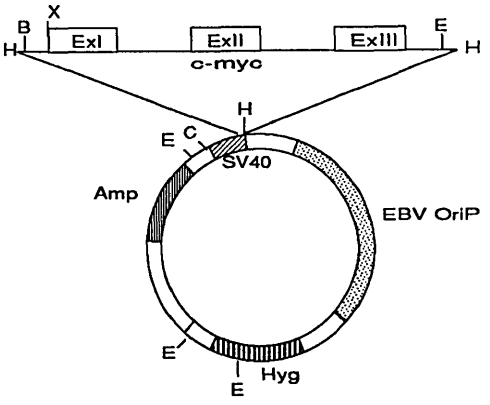
b) pHEBoSV



c) pHEBoSVmyc2,3



d) pHEBoSVmyc1,2,3



Not to scale

pSV3Neo taken from Southern and Berg (1982)
pHEBoSV, pHEBoSVmyc2,3 and pHEBomyc1,2,3 taken from Lombardi et al (1987)

1.2.3 pHEBoSVmyc2,3 (Lombardi *et al.*, 1987)

pHEBoSVmyc2,3 (figure 5c), contains exons II and III of the human c-*myc* gene inserted into pHEBoSV, in such a manner that the c-*myc* gene is driven from the SV40 enhancer/promoter region. Since the larger of the 2 species of c-*myc* (67kD) has its translational start codon in exon I (Hann *et al.*, 1988) only the smaller 64kD protein, with its start codon in exon II, is encoded by this construction.

1.2.4 pHEBoSVmyc1,2,3 (Lombardi, *et al.*, 1987)

pHEBoSVmyc1,2,3 was constructed in a manner similar to that of pHEBoSVmyc2,3, with all 3 exons of c-myc being inserted into pHEBoSV, under the control of the SV40 early region promoter (figure 5d).

pSV3Neo was a gift from Dr J. Burke (University of Sussex). pHEBoSV, pHEBoSVmyc2,3 and pHEBoSVmyc1,2,3 were gifts from Dr R. Dalla-Favera (New York University).

2. ESTABLISHMENT OF TRANSFECTED CELL LINES

2.1 Introduction

Numerous methods have been described for introducing DNA sequences into cells including electroporation (Potter, *et al.*, 1984), calcium phosphate/DNA precipitation (Graham and van der Ab, 1973), DEAE-dextran mediated transfection (McCutchan and Pagano, 1968), retroviral mediated transfection (for review see Brown and Scott, 1987), microinjection (Graessman *et al.*, 1980) and liposome mediated transfection (Felgner *et al.*, 1987). Calcium phosphate and DEAE-dextran methods are generally unsuitable for transfecting lymphoid cells (Potter *et al.*, 1984; Toneguzzo *et al.*, 1986). Microinjection has been used previously in this laboratory for introducing DNA into LCLs and cord blood lymphocytes but proved an inefficient and time-consuming method (Dr P. Smith, Royal Postgraduate Medical School, London, UK, personal communication). Retroviruses have been used, with some success, to transfect lymphoblastoid cells (Seremetis *et al.*, 1989); and recently liposome mediated transfection has been used to transfect a variety of cell types (Felgner and Ringold, 1989), using a positively charged lipid called DOTMA (N[1-2,3-dioleyloxy]propyl). This reagent has proved an efficient and convenient method

for introducing DNA into cells. However, the most widely used method is that of electroporation which has been shown to be a relatively efficient method for transfection of most types of cells, including lymphoid cells (Potter *et al*, 1984, Toneguzza *et al*, 1986; Knutson and Yee, 1988).

Initially, in these experiments, electroporation was used to introduce DNA into lymphoblastoid cells, using a "Gene Pulser" apparatus (BioRad Laboratories). Cells and DNA were placed in a plastic cuvette and electrical pulses of predetermined voltage and decay times delivered via electrodes attached to the cuvette, with a 0.4cm electrode gap (BioRad Laboratories). Before attempting to transfect the *myc*-containing plasmids into cells, two preliminary pieces of data were required. Firstly, it was necessary to determine optimal electroporation conditions; namely peak voltage (kV) and decay time of the electrical pulse, which is determined by the capacitance used (0.25-960μFd) (Knutson and Yee, 1987). Secondly, it was necessary to determine the concentrations of hygromycin B required to cause 100% cell death in cells not transfected with plasmids encoding the hygromycin B phosphotransferase gene. Having done this, transfection of cells with pHEBoSV, pHEBoSVmyc2,3 and pHEBoSVmyc1,2,3, and establishment of stable transfected, hygromycin B resistant cell lines could be attempted.

2.2 Experiments to determine optimal electroporation conditions

In preliminary experiments to determine optimal electroporation conditions 3 LCLs (CHEP, ELI and IB4) (Chapter V, section 7.6) were transfected with 10μg pSV3Neo plasmid DNA. Forty eight hours after transfection, cells were analysed for the presence of SV40 LT by indirect immunofluorescence (IF), using a rabbit polyclonal antisera against SV40 LT (115). A variety of voltage (KV) and capacitance (μFd) settings were used and results of staining for expression of the SV40 LT antigen are presented in Table 2. Low numbers of CHEP cells expressing SV40 LT (<0.1%) were detected using 3 different voltage and capacitance combinations (0.3kV, 125μFd; 0.35kV, 125μFd; 0.80kV, 25μFd). CHEP cells appeared comparatively fragile, with death of all cells a common result of electroporation. ELI LCL proved similarly resistant to electroporation, with low level expression of SV40 LT (<0.1%) being detected with 2 voltage and capacitance combinations (0.66kV, 25μFd; 0.70kV, 25μFd). Even this low frequency of LT antigen expression detected in CHEP and ELI LCLs proved difficult to repeat, and on subsequent occasions, using identical conditions, no expression of SV40 LT was detected.

Table 2

Transient expression of SV40 large T antigen in LCLs transfected with pSV3Neo.

10^6 cells from three different lymphoblastoid cell lines were electroporated with 10μg pSV3Neo DNA using a variety of different voltage (kV) and capacitance (μFD) combinations. Electroporated cells were cultured for 48 hours and stained, by indirect immunofluorescence, for expression of the pSV3Neo encoded SV40 large T (LT) antigen.

CHEP LCL			ELI LCL			IB4 LCL		
KV	μFD	LT	KV	μFD	LT	KV	μFD	LT
0.30	125	+	0.45	25	-	0.30	0.25	-
0.30	250	-	0.45	500	-*	0.30	1.0	-
0.30	500	-*	0.45	960	-*	0.30	3.0	-
0.30	960	-	0.50	25	-	0.30	25	-
0.35	125	+	0.66	25	+	0.30	125	-
0.35	250	-*	0.68	25	-	0.30	250	+
0.35	500	-*	0.70	25	+	0.30	500	++
0.40	125	-*	0.72	25	-*	0.30	960	+++
0.40	250	-*	0.74	25	-	0.70	25	-
0.40	500	-*	0.90	25	-	0.90	25	+
0.45	125	-*	1.10	25	-	1.10	25	++
0.45	250	-*	1.20	25	-	1.30	25	++
0.80	25	+	1.60	25	-	1.50	25	-
1.00	25	-	2.00	25	-	1.70	25	+
1.20	25	-				1.90	25	-
1.40	25	-				2.10	25	-*
1.60	25	-						

- No cells expressing SV40 LT
+ <0.1% of cells expressing SV40 LT
++ <1% of cells expressing SV40 LT
+++ >1% of cells expressing SV40 LT
* No viable cells after 48 hours

In contrast, IB4 cells could be successfully transfected by electroporation (Table 2), with more than 1% of cells expressing LT after pulsing with a number of voltage and capacitance settings (see for example figure 6). The voltage and capacitance settings (0.3kV, 960μFD) which resulted in successful transfection of IB4 cells were not successful when applied to ELI and CHEP LCLs.

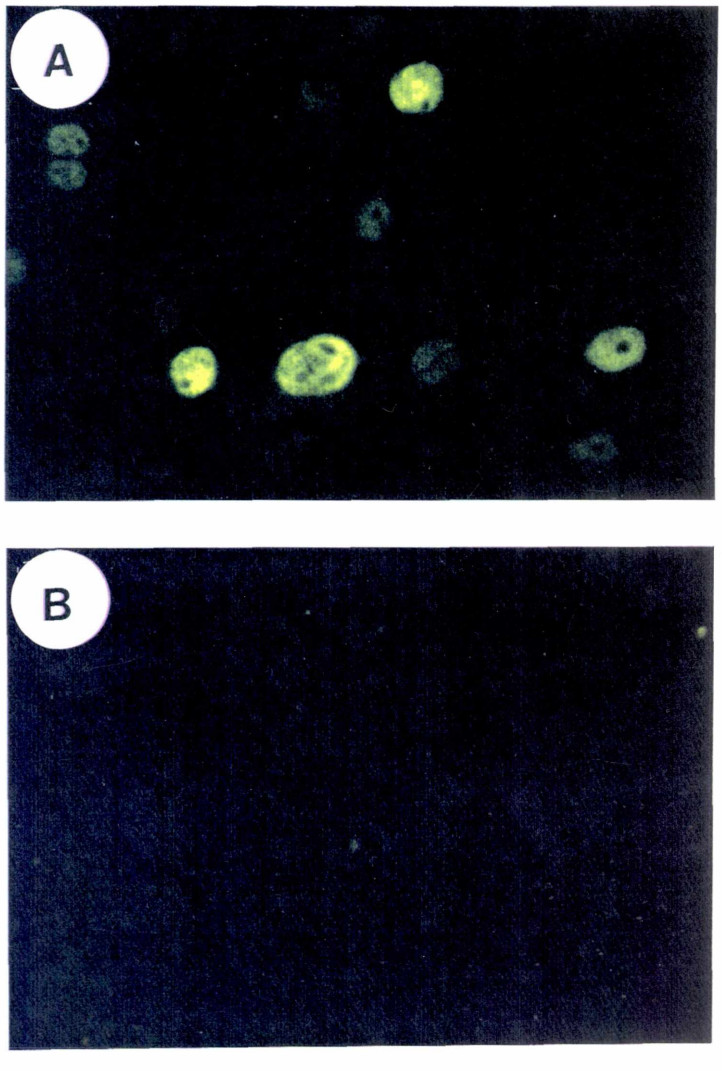
Stopper *et al* (1988) reported that using multiple electroporation pulses, with one minute intervals between pulses, increased efficiency of transfection in murine hybridoma cells. Similar experiments were attempted using ELI and IB4 cell, in which one to five pulses, with 60 second incubations on ice between pulses were applied to the cells, using voltage and capacitance combinations which had given expression of SV40 LT in previous experiments. In ELI cells administering more than one pulse (0.7kV, 25μFD) resulted in no viable cells after 48 hours. A single pulse resulted in no expression of SV40 LT, further underlining the problems of consistently transfecting these cells. A single pulse (0.3kV, 960μFD) applied to IB4 cells resulted in expression of SV40 LT in greater than 1% of cells, similar to the results noted earlier. Viability was high, with approximately 70% of cells excluding trypan blue 24 hours after transfection. Applying a second pulse resulted in a reduction of viability to approximately 15% and no expression of SV40 LT was observed. More than 2 pulses resulted in no viable cells being detected. These findings are in line with those of Knutson and Yee (1988), who also found that multiple pulses resulted in increased cell death, with no significant increase in numbers of cells expressing the transfected gene. The same authors also observed that optimal electroporation conditions varied between cell lines, and that expression of SV40 LT varied between experiments, despite using identical conditions. They also used different electroporation buffers, but found that normal growth medium was the most suitable.

2.3 Sensitivity of cell lines to hygromycin B

Experiments were performed to determine the concentrations of hygromycin B required to cause 100% cell death in cells not expressing the hygromycin B resistance gene. Various concentrations of hygromycin B (0 - 400μg/ml) were added to triplicate 0.2ml cultures of 10^4 cells. After 10 days in culture, growth in the presence of hygromycin B was assessed by adding tritiated thymidine for 4 hours, harvesting the cells and measuring incorporation of radioactive thymidine on a beta-particle counter. CHEP and ELI LCLs were relatively sensitive to hygromycin B requiring

Figure 6

Photomicrographs showing expression of SV40 large T antigen (LT) in IB4 cells transfected with pSV3Neo plasmid DNA.



IB4 cells were transfected with 10μg pSV3Neo DNA using electroporation (0.3kV, 960μFD). The pSV3Neo encoded SV40 large T antigen was detected by indirect immunofluorescence using a polyclonal anti-serum (115). Cells were examined on a U/V microscope at x400 magnification. A: pSV3Neo transfected cells stained with antibody against SV40 large T antigen. B: control un-transfected IB4 cells stained with antibody against SV40 large T antigen. The bar represents 50μm.

concentrations of 100μg/ml to inhibit proliferation (figure 7). In contrast, IB4 cells required a much higher concentration (400μg/ml) to completely inhibit proliferation. To confirm that these concentrations of hygromycin B were sufficient to cause 100% cell death in non-transfected cells, cells were stained with trypan blue after 10 days in culture with the relevant concentration of hygromycin B. No viable cells were detected. The concentrations of hygromycin B required to cause 100% cell death are similar to those reported by Sugden *et al* (1985) who looked at growth of various LCLs and BL cell lines in hygromycin B.

2.4 Establishment of stable transfected cell lines

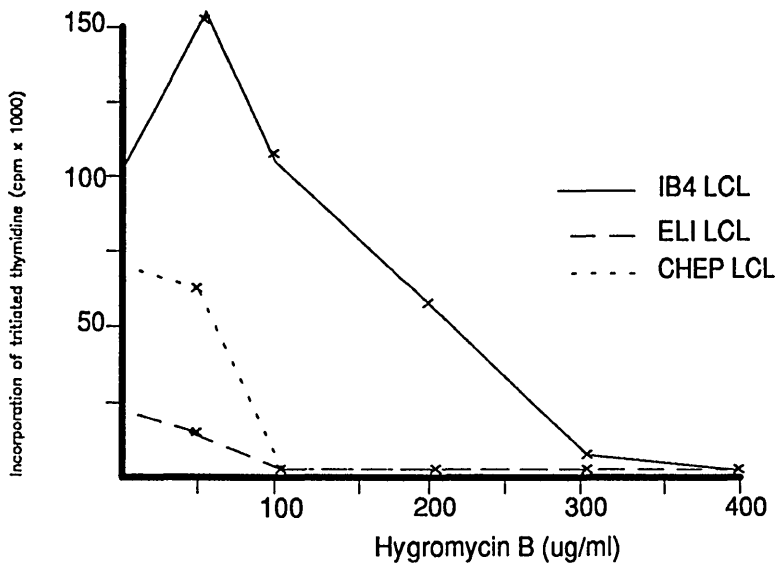
Experiments were performed in order to establish stable transfected cells using the pHEBoSV, pHEBoSVmyc2,3 and pHEBoSVmyc1,2,3 plasmid DNA. Forty-eight hours after electroporation hygromycin B was added to the relevant concentration. Cells were fed weekly, by replacing half the medium, until resistant cells began to proliferate. Proliferating cells were expanded in culture, and selection with hygromycin B maintained at all times.

Despite several attempts, no hygromycin B resistant cell lines were established following transfection of ELI and CHEP LCLs, using the voltage and capacitance settings which had resulted in expression of SV40 LT antigen in some transient assays. However, since the results obtained in the transient assays were highly variable, it is possible that the plasmids either were not being introduced into the cell lines, or were not being expressed once inside the cells.

A number of stable hygromycin B resistant cell lines were established after transfection of IB4 cells using a voltage of 0.3kV and a capacitance of 960μFD, and selection with hygromycin B at 400μg/ml. Proliferating foci of cells were generally observed within 14 days. Untransfected control cultures did not survive selection with hygromycin B. Three hygromycin B resistant pHEBoSV transfected cell lines [designated IB4-HEBo(30/3), IB4-HEBo(24/3), and IB4-HEBo(12/3)] were established. Two pHEBoSVmyc2,3 cell lines [(IB4-myc23(24/3) and IB4-myc23(17/1)] and 1 pHEBoSVmyc123 transfected cell line [IB4-myc123(24/3)] were also established following selection with hygromycin B. These cell lines were expanded and cultured in medium containing 400μg/ml hygromycin B.

Figure 7

Sensitivity of CHEP, ELI and IB4 cell lines to hygromycin B.



Triplicate 0.2ml cultures of 10^4 ELI, CHEP and IB4 lymphoblastoid cells were cultured for 10 days in normal growth medium containing 0, 50, 100, 200, 300 and 400µg/ml of hygromycin B. After 10 days cells were pulsed for 4 hours with 1µCi tritiated thymidine, harvested and incorporation of thymidine assessed on a beta counter. Data are the mean counts per minute (cpm) of the three replica cultures for each dilution of hygromycin B.

2.5 Cloning of IB4-myc23(24/3)

The hygromycin B resistant cell lines almost certainly arose from the outgrowth of more than one transfected cell. To examine clonality the cell lines were stained by direct IF for the presence of kappa and lambda Ig light chain expression. All transfected IB4 cell lines, in addition to the parental IB4 cell line, were exclusively lambda light chain positive, indicating that the parental IB4 cell line was probably clonal in origin.

The IB4-myc23(24/3) cell line was cloned using limiting dilution, by adding cells at concentrations of 1, 10 and 100 per well to round bottom microculture plate wells containing 10^4 irradiated peripheral blood mononuclear cells from an EBV seronegative donor as feeder cells. A total of 600 wells for each cell dilution were plated out and left for 4 weeks. Cloning was carried out in growth medium containing 20% FCS without hygromycin B (Crawford, 1986). In the plates with 100 IB4-myc23(24/3) cells per well over 50% of wells showed proliferation. At a dilution of 1 cell per well no proliferating cells were observed. In the plates with 10 cells per well, 48 out of 600 wells contained proliferating foci of cells, and these were considered clonal. However, since the parental IB4 cell line was clonal, the clonality of these transfectants could not be confirmed. The proliferating cells were fed with fresh medium as required and expanded into flat-bottom microculture plates, 15mm culture wells and finally into 50ml cell culture flasks. Once in the flasks, selection with hygromycin B was resumed. Of the original 48 clones only 7 survived selection with hygromycin B at 400μg/ml (clones 5, 6, 7, 27, 39, 46 and 47). This suggests that during the cloning procedure, in the absence of hygromycin B (4-6 weeks, depending on the clone), the majority of the cells which formed the clones lost, or ceased expressing, the hygromycin B resistance gene. This phenomenon, in which plasmids are lost from cells not maintained in selection, is not uncommon in cells transfected with this plasmid (Dr. B. Sugden, University of Wisconsin, USA, personal communication). The importance of maintaining selection is underlined by this experiment.

2.6 Mycoplasma

At various stages during the experiments presented here, cell lines were tested for the presence of mycoplasma contamination using a commercial detection system (MycoTect, Gibco). Mycoplasma detection was performed as part of a routine laboratory service by Mr K. Patel and Ms N. Syed (Department of Virology, RPMS). Initially, no mycoplasma contamination was detected in the

transfected IB4 cell lines. However, subsequent testing revealed low, but detectable, levels of mycoplasma contamination in all transfected IB4 cell lines. The contamination became more apparent when the cells were cultured in the absence of hygromycin B for 2-3 weeks, suggesting that hygromycin B, had suppressed, but not erradicated, the mycoplasma infection. It is not known at what stage cell lines became contaminated, but it is likely that the parental IB4 cell line was the source of contamination, since subsequent testing of IB4 cells from our laboratory revealed contamination with mycoplasma. Similarly, IB4 cells from other laboratories were contaminated with mycoplasma when tested. As far as it was possible to detect, the level of contamination was similar in all transfected cell lines. Wherever it was thought that mycoplasma contamination might influence results, such as in experiments involving incorporation of tritiated thymidine, alternative experiments were performed to confirm findings. All other cell lines used in these experiments were mycoplasma free.

2.7 Summary

Despite several attempts it proved impossible to establish hygromycin B resistant CHEP and ELI LCL following transfection with the *myc*-containing plasmids. These 2 cell lines were the ideal candidates for transfection with c-*myc* as paired BL cell lines were available for comparison (Rooney *et al.*, 1985). It is not unusual for LCLs to be resistant to transfection (Dr. J. Knutson, University of Wisconsin, USA, personal communication). In the absence of transfected ELI or CHEP LCLs, the IB4 LCL was used. Its advantages were, firstly that it was relatively easy to transfect with a high degree of consistency and secondly, it is a clonal cell line and potentially represents a more discrete population of B cells than other polyclonal EBV immortalised cell lines. The disadvantages were that there was no paired BL cell line with which to compare and also, as became apparent later in the study, the cell line was contaminated with mycoplasma.

3. EXPRESSION OF TRANSFECTED C-*MYC* GENES IN IB4 CELLS

3.1 Introduction

Having established hygromycin B resistant cell lines it was important to establish that the transfected c-*myc* gene was being expressed. This was achieved by using the S1 endonuclease protection assay to distinguish between endogenous and exogenous, plasmid derived, c-*myc* transcription. In addition, levels of c-Myc protein were examined by immunoblotting, and using an ELISA

assay.

3.2 Transcription of c-*myc*

To examine transcription of the transfected c-*myc* gene and compare it to that of the endogenous gene in both c-*myc* and pHEBoSV-transfected cell lines, S1 endonuclease mapping of RNA transcripts from IB4-HEBo(30/3) and IB4-myc23(24/3) was performed. S1 endonuclease acts by digesting single stranded nucleic acid. By hybridising a ^{32}P labelled single stranded DNA probe, spanning intron I and exon II of c-*myc*, to RNA extracted from IB4-HEBo(30/3) and IB4-myc23(24/3) cells, endogenous and exogenous (plasmid derived) transcripts could be differentiated.

The probe (M13myc.i1.e2) was generated from a longer c-*myc* M13 clone (M13JI71.5Sac, gift from T. Rabbitts, University of Cambridge; Hamlyn and Rabbitts, 1983) as outlined in figure 8. The full length M13 clone was digested with PstI and allowed to re-ligate with the result that a 762bp PstI-PstI fragment from intron I was lost. The re-ligated M13 DNA was transfected into competent bacteria and template M13 purified. This template was used to generate a 472bp radiolabelled ssDNA probe, complementary, in part, to the c-*myc* transcript, to be generated by primer extension with ^{32}P labelled nucleotide, and digestion with PstI and SacII. When hybridised to probe DNA, endogenous c-*myc* transcripts, with a normal exon I-exon II splicing pattern, protect a short fragment of the probe (splice acceptor - PstI = 138bp). pHEBoSVmyc2,3 transcripts, which do not have the normal exon I-exon II splice pattern, but instead include a portion of the first intron linking the SV40 early region to exon II, protect a longer fragment of probe from the action of S1 endonuclease (AluI-PstI = 253bp).

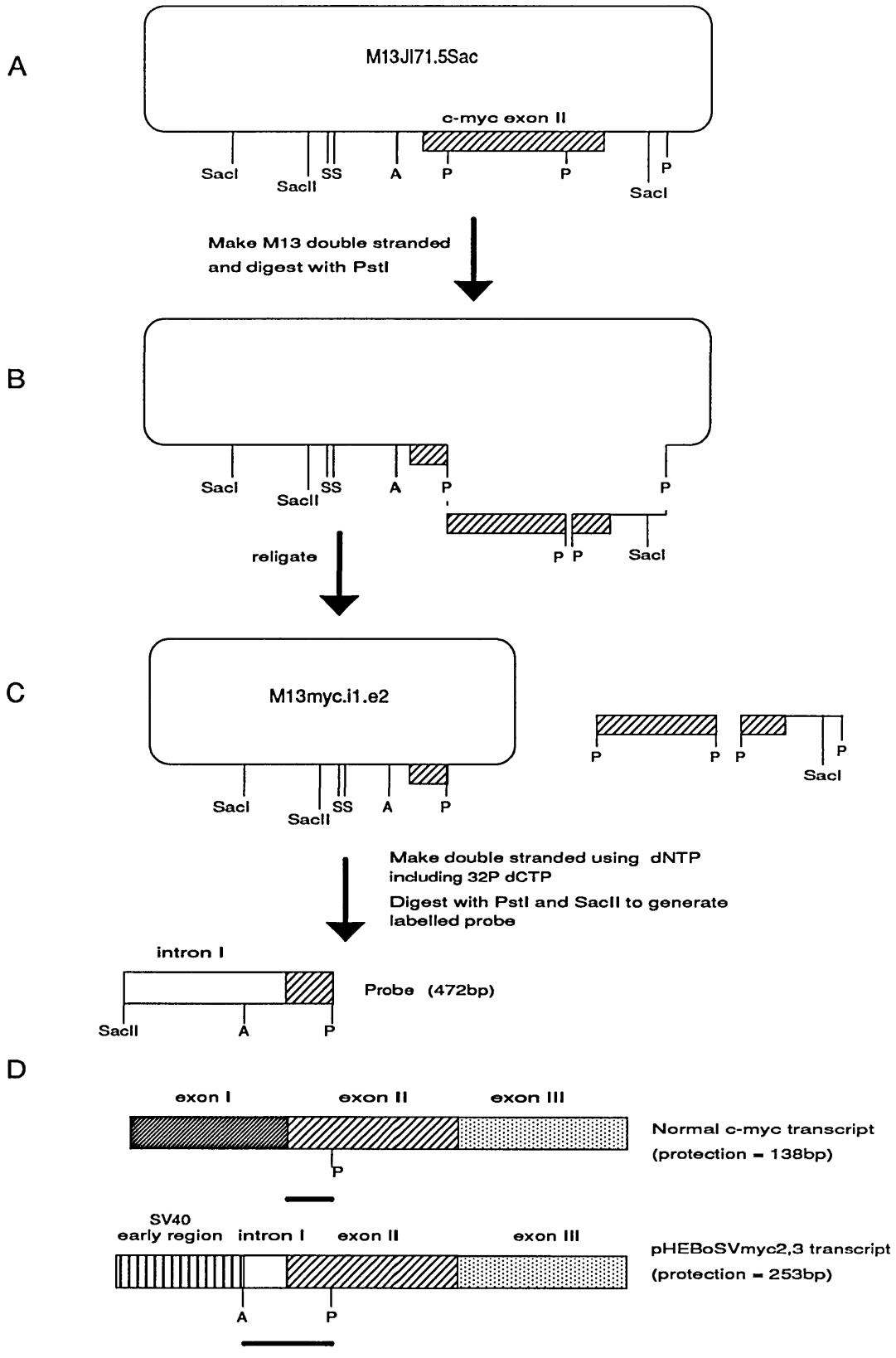
The ^{32}P labelled probe was hybridised to 20μg total RNA from IB4-HEBo(30/3) and IB4-myc23(24/3), digested with 100 units of S_1 endonuclease, run on a high resolution denaturing gel (5% acrylamide, 8M urea) and the dried gel exposed to X-ray film. Probe hybridised to template M13 and yeast tRNA were included as controls. Markers were 1μg pBR322 digested with MspI and end-labelled with ^{32}P dCTP. Results of this experiment revealed a considerable increase in levels of c-*myc* mRNA in the IB4-myc23(24/3) cell line, relative to the IB4-HEBo(30/3) control cells (figure 9, compare tracks 2 and 3). The slightly larger than predicted size of endogenous transcripts is probably a consequence of differing migratory rates for double stranded DNA markers and DNA-RNA hybrids (Dr. M. Jones, Royal Postgraduate Medical School, personal communication;

Legend: Figure 8

Construction of M13myc.i1.e2 single stranded DNA probe for S1 endonuclease protection assay

Diagram showing construction of M13myc.i1.e2. The parent M13 c-*myc* clone (M13JI71.5Sac) containing part of intron I and all of exon II (SacI-SacI) (figure A) was digested with PstI (P) (figure B) and allowed to religate with the result that a 762bp PstI-PstI fragment was lost (figure C). This resulted in an M13 template containing a 472bp SacII-PstI fragment of c-*myc* spanning intron I and exon II. Primer extension in the presence of ^{32}P dCTP and digestion with SacII and PstI resulted in the generation of a radio-labelled probe complementary to the 5' end of exon II and the 3' end of intron I of c-*myc* (figure D). The thick horizontal lines in figure D indicate regions of endogenous and pHEBoSVmyc2,3 transcripts protected from the actions of S1 endonuclease by the probe. S - SmaI; P - PstI; A - AluI; dNTP - deoxynucleotide triphosphates; dCTP - deoxycytidine triphosphate.

Figure 8

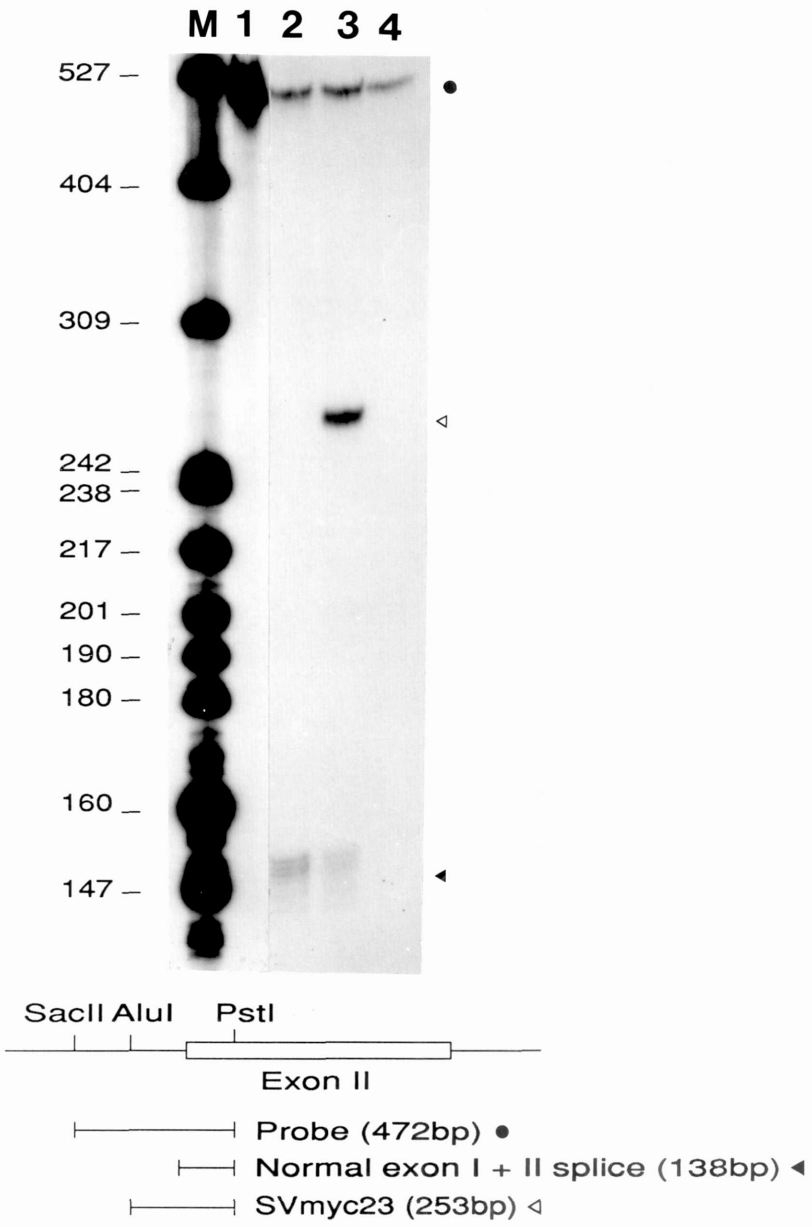

A
M13JI71.5Sac
c-myc exon II
SacI
SacII
SS
A
P
P
P
SacI
Make M13 double stranded
and digest with PstI
B
SacI
SacII
SS
A
P
P
P P
SacI
religate
C
M13myc.i1.e2
SacI
SacII
SS
A
P
P P P P
SacI
Make double stranded using dNTP
including 32P dCTP
Digest with PstI and SacII to generate
labelled probe
intron I
SacII
A
P
Probe (472bp)
D
exon I
exon II
exon III
P
Normal c-myc transcript
(protection = 138bp)
SV40
early region
intron I
exon II
exon III
A
P
pHEBoSVmyc2,3 transcript
(protection = 253bp)

Legend: Figure 9

S1 endonuclease mapping of RNA transcripts from IB4-HEBo(30/3) and IB4-myc23(24/3).

S1 endonuclease mapping of 20μg of total RNA extracted from IB4-HEBo(30/3) and IB4-myc23(24/3) cell lines. RNA was hybridised to a single stranded DNA probe which allows endogenous c-*myc* transcripts with a normal exon I/exon II splicing pattern, to be distinguished from exogenous pHEBoSVmyc2,3 derived c-*myc* transcripts. Lane 1, unhybridised probe; lane 2, IB4-HEBo(30/3); lane 3, IB4-myc23(24/3); lane 4, yeast tRNA. Positions of plasmid-derived (◁) and endogenous (◀) transcripts are indicated, along with unhybridised probe (●). Markers (M) are 1μg pBR322 DNA digested with MspI and end-labelled with ^{32}P.

Figure 9


M 1 2 3 4
527
404
309
242
238
217
201
190
180
160
147
SacII AluI PstI
Exon II
Probe (472bp) ●
Normal exon I + II splice (138bp) ◀
SVmyc23 (253bp) ◁

Lombardi *et al.*, 1987). The increase in c-*myc* mRNA was entirely due to transcription from the transfected plasmid, as endogenous transcription appeared partially suppressed.

3.3 Protein expression

To examine whether increased transcription of c-*myc* in IB4-myc23(24/3) cells resulted in similarly increased levels of protein, immunoblotting of protein extracted from IB4 transfectants was performed. Protein from 2 x 10^6 cells was separated by SDS-PAGE on a 10% acrylamide gel. After electrophoretic transfer to nitrocellulose c-Myc protein was detected using a monoclonal antibody (mAb) (Myc1-9E10).

As demonstrated in figure 10a, the increased levels of c-*myc* transcription in the IB4-myc23(24/3) cell line were reflected in significantly increased levels of c-Myc protein (64kDa) relative to IB4-HEBo(30/3). In addition the IB4-myc123(24/3) cell line had similarly increased levels of protein. However, none of the myc-transfected cell lines express levels of c-Myc comparable to the BL cell line, Raji. These findings were confirmed on at least 3 separate occasions, and on separately established IB4-HEBo and IB4-myc23 cell lines.

A faint band can be seen in IB4-myc123(24/3), migrating just above the large band. This probably corresponds to the larger (67KD) of the two c-Myc proteins reported by Hann and Eisenman (1984) which appears to start from a novel start codon (CTG) at the 3' end of exon I (Hann *et al.*, 1988). The lower band corresponds to the 64kD protein which starts from a conventional ATG start codon at the start of exon II, and has been found in excess over the 67kD protein in LCLs (Hann and Eisenman, 1984).

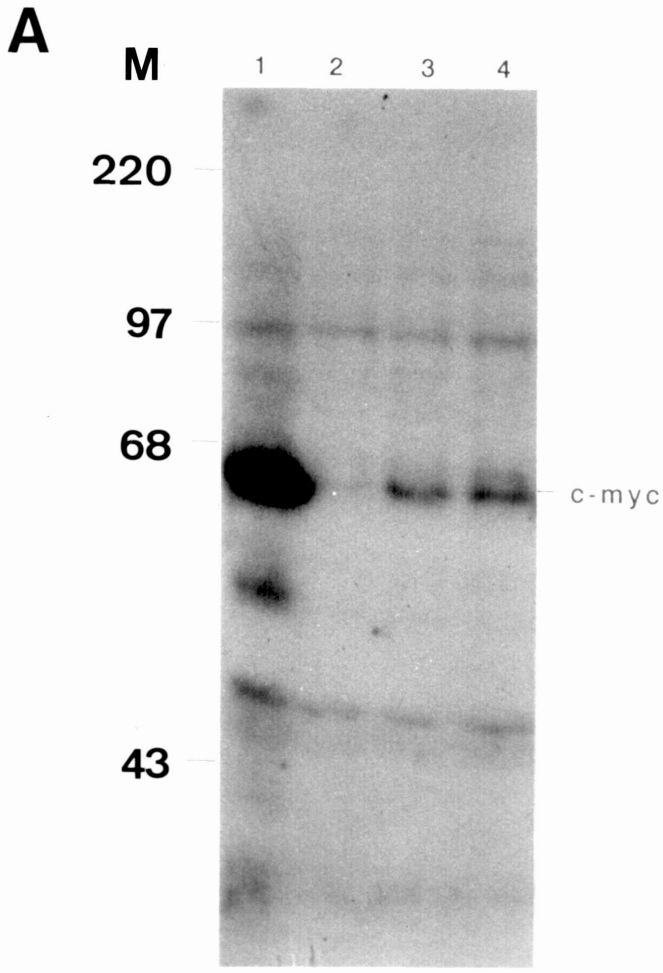
The bands visible at approximately 100kD and 47kD have been observed previously and are probably cross-reactions with other proteins (Evan *et al.*, 1985; Dr. G. Evan, Imperial Cancer Research Fund, personal communication). A protein of approximately 55kD is also detected using this antibody in Raji cells, but apparently not in IB4 cells. The identity, or significance, of this protein is not known.

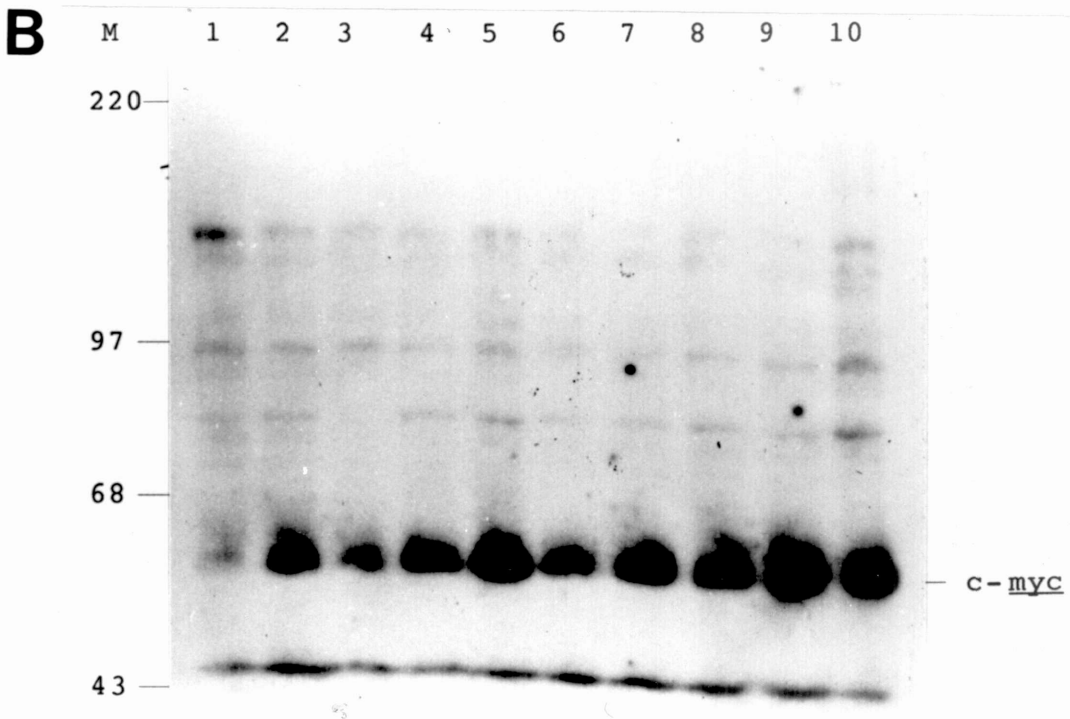
Immunoblot analysis of c-Myc protein expression in 7 clones derived from the IB4-myc23(24/3) cell line revealed similarly increased levels of protein in all clones (figure 10b), compared to IB4-HEBo(30/3), although some variation between clones was observed (figure 10b, compare clone 5 with clone 46).

Legend: Figure 10

Immunoblot analysis of c-Myc protein expression in IB4 transfectants

Protein extracted from 2 x 10^6 cells was separated on a 10% polyacrylamide gel in the presence of 0.1% SDS, transferred to nitrocellulose and probed with a c-Myc specific monoclonal antibody (Myc1-9E10). A: Lane 1, Raji (BL cell line); lane 2, IB4-HEBo(30/3); lane 3, IB4-myc23(24/3); lane 4, IB4-myc123(24/3); M, high molecular weight protein standards. B: lane 1, IB4-HEBo(30/3); lane 2, IB4-myc23(24/3); lane 3, IB4-myc23(17/1); lane 4, IB4-myc23(24/3) clone 5; lane 5, IB4-myc23(24/3) clone 6; lane 6, IB4-myc23(24/3) clone 7; lane 7, IB4-myc23(24/3) clone 27; lane 8, IB4-myc23(24/3) clone 36; lane 9, IB4-myc23(24/3) clone 46; lane 10, IB4-myc23(24/3) clone 47; M, high molecular weight protein standards.


A
M
1
2
3
4
220
97
68
43
c-myc


B
M
1
2
3
4
5
6
7
8
9
10
220
97
68
43
c-myc

To confirm these findings, and attempt to quantify levels of protein, an ELISA for c-Myc protein was performed (Moore *et al.*, 1987). In this assay, c-Myc protein is captured by an antibody bound to an ELISA plate which recognises a peptide sequence common to all identified Myc proteins (Moore *et al.*, 1987). Bound c-Myc protein is detected by incubating with a c-*myc*-specific antibody conjugated to alkaline phosphatase, and a colour reaction developed by adding a sensitive enzyme amplifying system (AMPAK, IQ (Bio) Ltd). Comparison to a bacterially produced protein standard (Watt *et al.*, 1985) allows quantification of c-Myc in terms of numbers of molecules per cell.

Results using protein lysates (from 2 x 10^5 cells) of IB4-HEBo(30/3), IB4-myc23(24/3) and the 7 clones of IB4-myc23(24/3) are presented in figure 11. For comparison protein lysate from 10^5 Raji cells was included. Values for OD_{492} varied between 0.042 for IB4-HEBo(30/3) and 0.167 for Raji cells. OD_{492} values for 5 negative controls, with no protein lysate, were between 0 and 0.004. Titrating the bacterial c-*myc* standard from 125pg to 15.6pg allowed a standard curve to be constructed (figure 11a). From this, the pg equivalents of c-Myc protein and the number of c-Myc molecules per cell calculated. It is known that 1ng c-Myc from 2 x 10^5 cells is equivalent to 60000 molecules per cell (Moore *et al.*, 1987). The OD_{492} and amount of protein for each cell line, along with its equivalent, in terms of number of molecules per cell, is presented in figure 11b. From these data it appears that the levels of c-Myc protein in IB4-myc23(24/3) (an average of 3660 molecules per cell) are approximately three fold higher compared to IB4-HEBo(30/3) (1140 molecules per cell). Levels of protein vary in the clones of IB4-myc23(24/3) with some expressing higher levels of protein than the parental *myc*-transfected cell line, and others lower amounts (for example, compare clone 46 with clone 5). The levels of c-Myc protein in the BL cell line, Raji (13850 molecules/cell), were approximately 10 fold higher than IB4-HEBo(30/3) and 3-4 fold higher than in the IB4-myc23(24/3) cell line. These relative differences appeared to correlate well with the immunoblot data, although the differences between the clones were not so apparent. A previous study examining levels of c-Myc protein, using this assay, in various cell lines reported considerably higher levels of protein in Raji cells (Moore, *et al.*, 1987). The reasons for the differences between their findings, and those of this study, may simply be due to the inter-experimental variation noted by Moore *et al* (1987). The same authors, however, observed that the relative differences, between cell lines, in levels of protein remained constant. Alternatively, it may

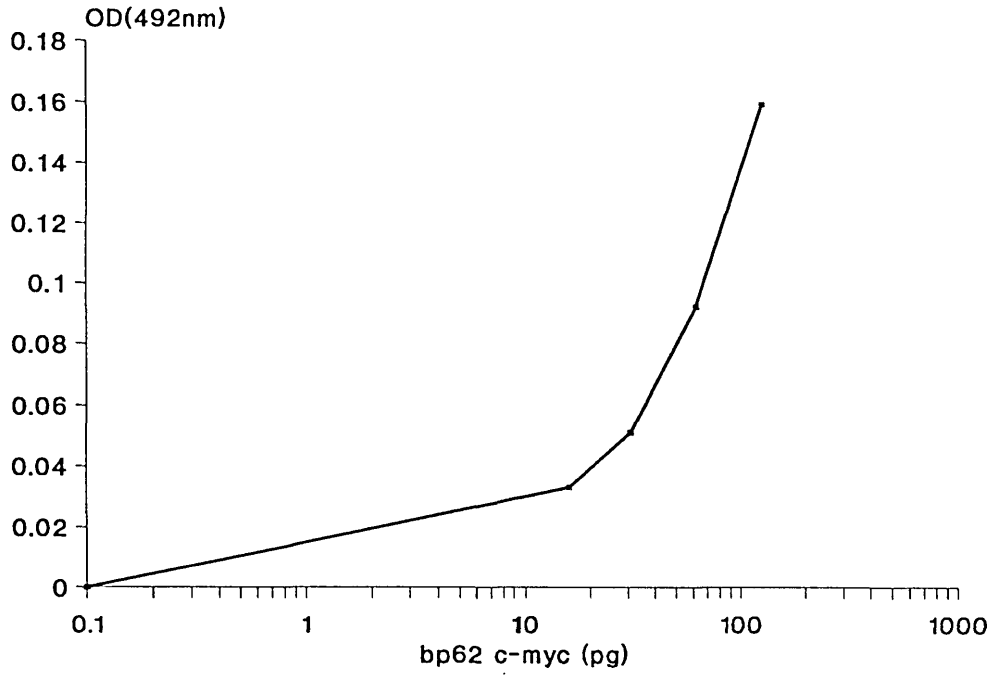
Legend: Figure 11

Amounts of c-myc protein per cell in IB4 transfectants as determined by ELISA.

The amount of c-Myc protein per cell was quantified using an ELISA in which protein extracted from 2 x 10^5 cells was incubated with anti-Myc antibody, bound to an ELISA plate. Bound protein was visualised by incubation with a peroxidase conjugated anti-c-Myc antibody, developing with an enzyme amplifying system and reading the optical density of each well at 492nm (OD_{492}). As a standard, bacterially produced c-Myc protein (bp62 c-Myc) was titrated from 125 - 15.6pg and included in the ELISA. Using this, a standard curve could be calculated (figure A). From the standard curve, average numbers of c-Myc molecules per cell were calculated (1ng c-Myc protein from 2 x 10^5 cells = 60000 molecules per cell, Moore *et al.*, 1987) Results obtained, comparing average numbers of molecules per cell from IB4-HEBo(30/3), IB4-myc23(24/3) and the seven sub-clones of IB4-myc23(24/3) with the BL cell line Raji are presented in figure B. (* - protein from 10^5 Raji cells was analysed).

Figure 11

A:



B:

Cell line	OD(492)	[protein] pg	Molecules/cell
IB4-HEBo(30/3)	0.042	19	1140
IB4-myc23(24/3)	0.087	61	3660
IB4-myc23			
clone 5	0.066	43	2580
clone 6	0.055	33	1980
clone 7	0.066	43	2580
clone 27	0.079	56	3360
clone 36	0.091	63	3780
clone 46	0.104	72	4300
clone 47	0.055	43	1980
Raji	0.167*	115	13850

be due to variation in reagents, materials or protein standard.

3.4 Summary

S1 endonuclease protection demonstrated clearly that the exogenous, plasmid derived, c-*myc* gene was being transcribed at high levels relative to the control IB4-HEBo(30/3) cell line. These data confirm the findings of Lombardi *et al* (1987) and provide further evidence that the SV40 enhancer/promoter region functions well in lymphoblastoid cells. The suppression of endogenous c-*myc* transcription seen in these experiments has also been observed by others (Adams *et al*, 1985; Lombardi *et al*, 1987). The increase in c-*myc* transcription seen in IB4-myc23(24/3) resulted in a significant increase in protein expression as determined by immunoblotting. Similarly high levels of protein were also seen in IB4-myc123(24/3) and clones derived from IB4-myc23(24/3). These findings were confirmed using a c-Myc ELISA. In none of these cell lines however, did levels of protein reach that seen in the BL cell line, Raji.

4. GROWTH CHARACTERISTICS OF IB4 CELL LINES CONSTITUTIVELY EXPRESSING C-*MYC*

4.1 Introduction

To determine whether constitutive expression of c-*myc* had any effect on the growth characteristics of IB4 cells, 4 different parameters of cell growth were examined; cell morphology, growth pattern, growth kinetics of cells in normal medium and growth in reduced serum concentrations.

4.2 Morphology

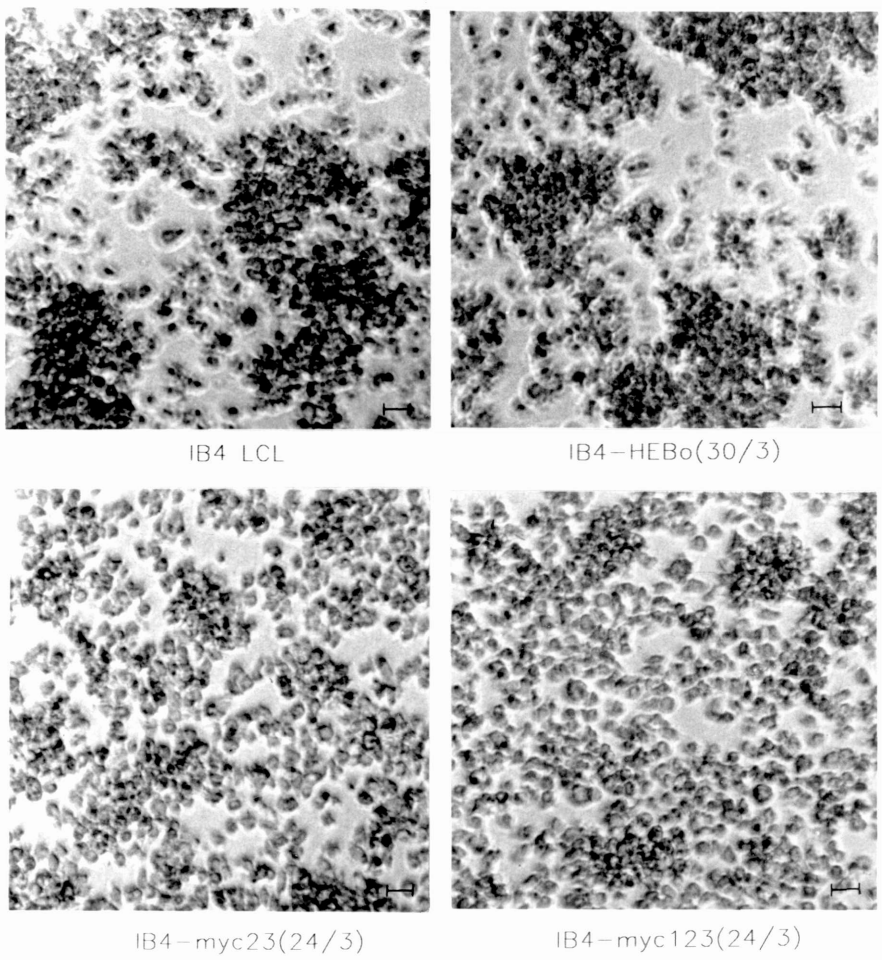
No difference in cell morphology was observed using a light microscope, with both the *myc*-transfected and control cell lines displaying the irregular morphology typical of lymphoblastoid cells.

4.3 Growth pattern

IB4-HEBo(30/3) cells, in culture, grew in large clumps typical of the parent IB4 cell line and other LCLs. These clumps started to form within 2 hours of the cells being resuspended to single cell suspension. In contrast, IB4-myc23(24/3) and IB4-myc123(24/3) cells demonstrated markedly less homotypic cell adhesion, and whilst some cell aggregation was observed after 2 hours the clumps of cells tended to be smaller and more cells were in single cell suspension (figure 12).

Figure 12

Growth pattern of parental IB4 cells and transfected IB4 cells.



Photomicrographs illustrating growth pattern of IB4 LCL, IB4-HEBo(30/3), IB4-myc23(24/3) and IB4-myc123(24/3) in normal growth medium. 2 x 10^5 cells were suspended to single cell suspension, left for 2 hours at 37°C, and photographed on an inverted light microscope at x200 magnification. Scale bar represents 50μm.

4.4 Growth kinetics

The growth kinetics of the transfected cell lines were assessed by resuspending cells at a density of 2 x 10^5 cells/ml in fresh medium and culturing for 18 days, without replacement of medium. Viability was assessed by counting cells in the presence of trypan blue and results are the mean of 3 separate experiments (figure 13). IB4-HEBo(30/3) cells had a doubling time of 2 days and reached a maximum cell density of 5.54 x 10^5 cells/ml by day 3. IB4-myc23(24/3) and IB4-myc123(24/3) had slightly faster doubling times (1-2 days). Both *myc*-transfected cell lines reached maximal densities after 4 days which were higher than IB4-HEBo(30/3) [IB4-myc23(24/3), 7.52 x 10^5/ml; IB4-myc123(24/3), 7.55 x 10^5/ml]. After 7 days, all 3 cell lines had reached a density of 4-5 x 10^5/ml, and by day 18, when the experiment was ended, the number of viable cells had only slightly declined (to approximately 3 x 10^5/ml) from the day 7 levels.

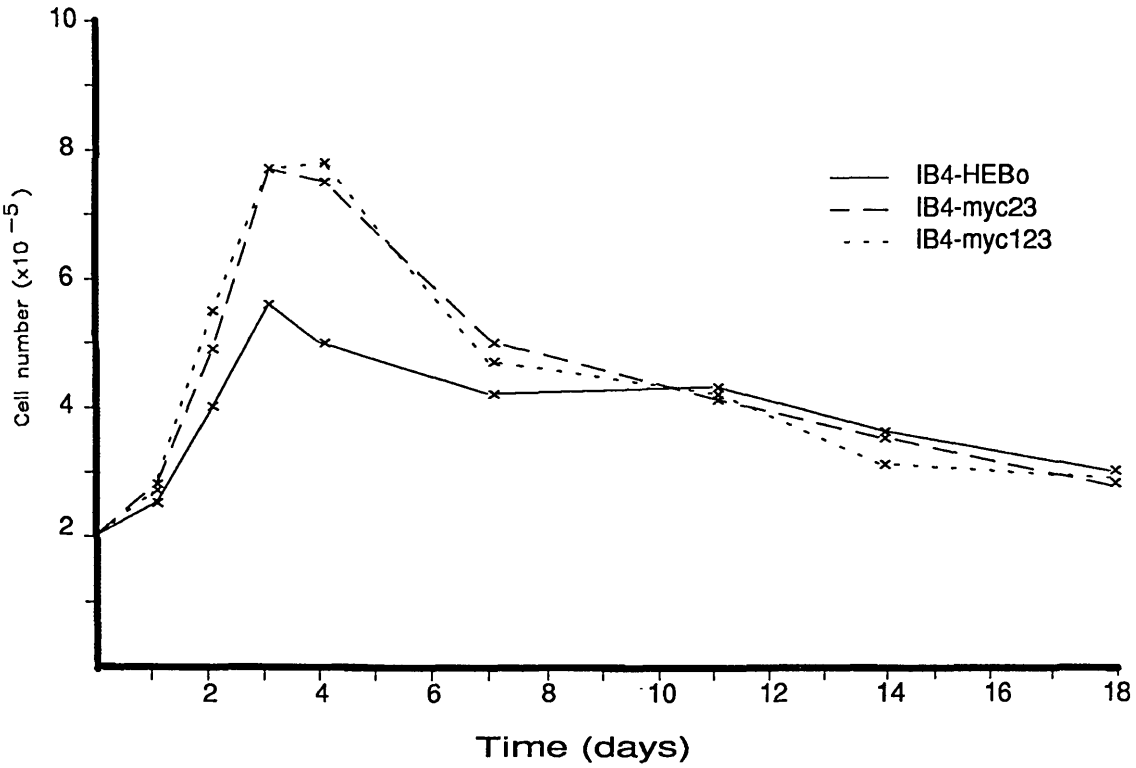
4.5 Growth in low serum concentrations

Growth of the transfected IB4 cell lines in low serum concentrations was assessed using 2 methods. Firstly, the cells were cultured in medium containing 1% FCS for 48 hours, and incorporation of tritiated thymidine during a 4 hour period measured. Results, presented in figure 14, are the mean and standard error of 3 separate experiments, with the exception of IB4-myc23(24/3) which is the mean of 2 experiments. Both IB4-myc23(24/3) (mean cpm = 47193) and IB4-myc123(24/3) (mean cpm = 48200) incorporated significantly more thymidine than the control cell line, IB4-HEBo(30/3) (mean cpm = 16178). Thus both *myc*-transfected cell lines proliferate at a higher rate than the control cell line, as assessed by DNA synthesis.

Because of the concern that mycoplasma might incorporate thymidine, and thus influence the data obtained above (section 2.6), an additional experiment was carried out. Cell proliferation in 1% FCS was assessed by counting the number of viable cells at 24 hour intervals for 4 days. Cells were fed daily, with half the medium being removed and replaced with fresh medium. The 2 *myc*-transfected cell lines were seen to increase in cell number whereas the control cell line showed little increase in cell number over 4 days (figure 15). From these data it is apparent that the *myc*-transfected cells have a greater proliferative capacity in reduced serum concentrations, possibly reflecting a reduced requirement for exogenous growth factors.

Figure 13

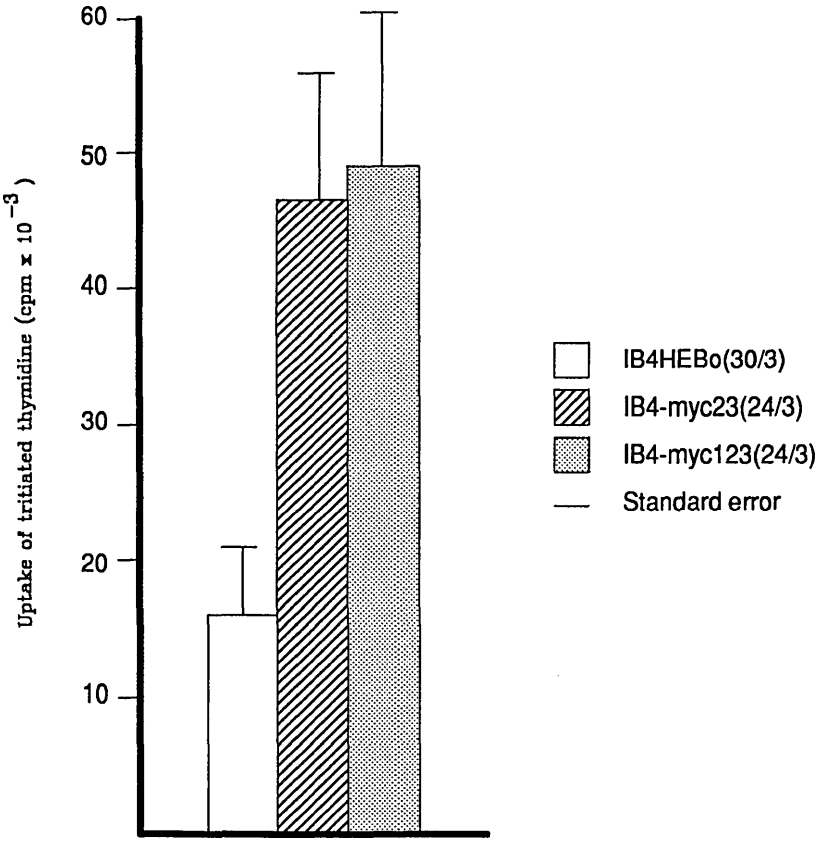
Growth of IB4-HEBo(24/3), IB4-myc23(24/3) and IB4-myc123 in normal growth medium



Growth of IB4-HEBo(30/3), IB4-myc23(24/3) and IB4-myc123(24/3) was assessed by resuspending cells in normal growth medium at a density of 2 x 10^5/ml and culturing for 18 days, with no addition of fresh medium. Cell proliferation was assessed by counting viable cells in the presence of trypan blue at regular intervals. Results are the mean of three experiments.

Figure 14

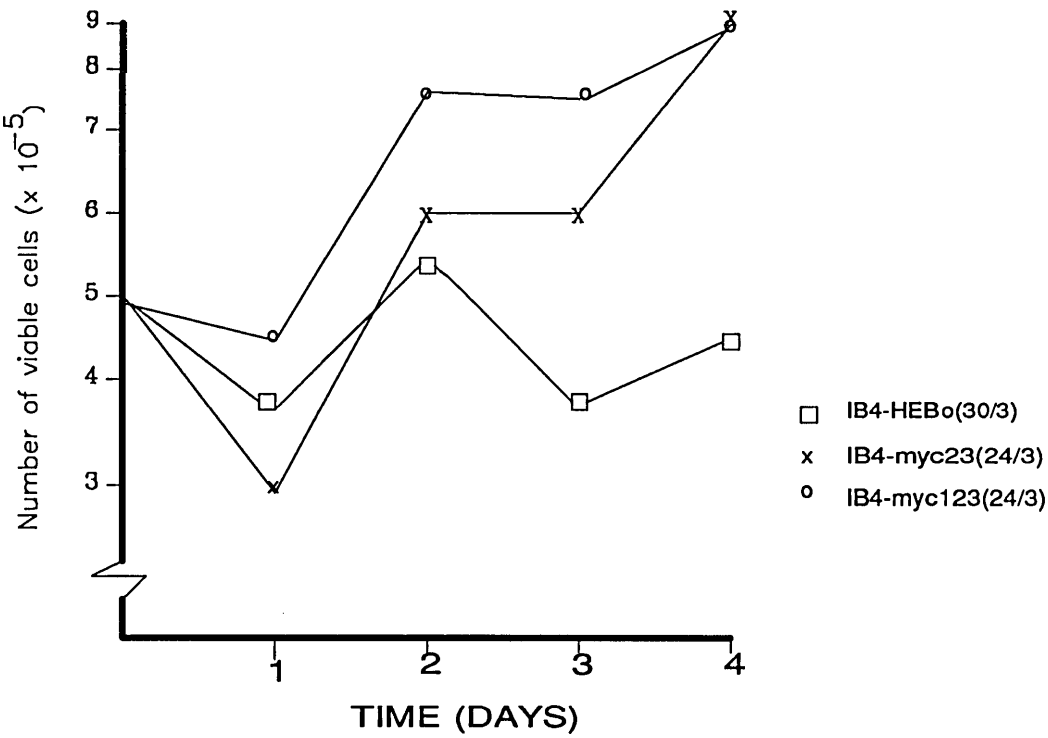
Uptake of tritiated thymidine by IB4 transfectants cultured in low serum concentrations



Proliferation of IB4-HEBo(30/3), IB4-myc23(24/3) and IB4-myc123(24/3) in low serum concentrations was assessed by culturing cells for 48 hours in growth medium containing 1% foetal calf serum and measuring incorporation of tritiated thymidine during a 4 hour period. Results are the mean of three experiments, with the exception of IB4-myc23(24/3) which is the mean of two experiments.

Figure 15

Growth of IB4 transfectants in low serum concentrations



Proliferation of IB4-HEBo(30/3), IB4myc23(24/3) and IB4-myc123(24/3) in low serum concentrations was assessed by culturing cells in growth medium containing 1% foetal calf serum for 4 days. Cells were counted daily in the presence of trypan blue to exclude dead cells.

4.6 Summary

In terms of growth characteristics, major differences between the control and *myc*-transfected cell lines were observed. Whilst the morphology of the cell lines appeared unchanged both IB4-myc23(24/3) and IB4-myc123(24/3) demonstrated markedly less homotypic cell aggegration in culture growing more in small clumps and single cell suspension, in a manner more similar to that seen in BL cells (Rooney *et al.*, 1986).

Both *myc*-transfected cell lines showed enhanced proliferation in low serum concentrations, suggesting a reduced dependence on exogenous growth factors. In normal growth medium, containing 10% FCS, the *myc*-transfected cell lines had a slightly faster doubling time, and reached a higher cell density than the control cell line. Interestingly, after 18 days in culture without replacement of medium, cells from all three lines remained viable, with an apparently stable cell density of approximately 3 x 10^5/ml.

5. EXPRESSION OF CELL SURFACE MOLECULES

5.1 Introduction

The expression of B cell specific surface molecules, including markers of B cell differentiation, was examined to determine the phenotype of the *myc*-transfected cells relative to control cells. The characteristics of the molecules examined are described in chapter I, section 3.2. Expression of HLA class I and DR molecules, and the cell adhesion molecules, LFA-1 (composed of an alpha chain [CD11a] and a beta chain [CD18]), LFA-3 (CD58) and ICAM-1 (CD54) was also examined (chapter I section 3.3). Finally expression of CD71, the transferrin receptor, was assessed.

Analysis of cell surface molecule expression was carried out by indirect immunofluorescence using monoclonal antibodies. Analysis of staining was generally carried out on an EPICS PROFILE flow cytometer (Coulter Electronics). Expression of cell surface molecules was determined by recording the mean linear fluorescence (FL) of 5000 cells in the presence of antibody. To correct for slightly differing cell sizes between cell lines the mean fluorescence intensity/size (FL/FS) ratio was calculated. Cell size was measured by forward light scatter (FS) and is proportional to cell volume. On the EPICS PROFILE FL/FS is calculated automatically for each cell.

Staining with some antibodies was analysed on a EPICS C SYSTEM fluorescence activated cell sorter (FACS). In these cases the mean FL for 25000 cells was recorded and normalised for size using the mean FS of the entire population of cells. Due to the differences in analyser and method of calculating FL/FS values the results obtained on the FACS are not directly comparable to those from the PROFILE, but are equally valid in determining relative differences in antigen expression between cell lines.

Unless stated otherwise results are the mean and standard error of three separate experiments. Details of antibodies used in these experiments, and the staining procedures are given in Chapter V (section 8) and Chapter VI (section 12).

5.2 Expression of B cell surface molecules (Table 3)

IB4-HEBo(30/3), IB4-myc23(24/3) and IB4-myc123(24/3) cell lines expressed low levels of CD10 (cALLa) and CD77 (BLA), but no significant difference in mean fluorescence intensity between c-*myc*- and pHEBoSV-transfected cell lines was noted (Table 3a, figure 16).

When the means of three separate experiments were taken, increases in expression of CD19, CD21 and CD23 were observed in *myc*-transfected cells relative to the control cells (Table 3a). However, in the case of CD19 and CD21, inter-experimental variation in relative levels of expression was observed (figure 16). Expression of CD19 was increased approximately 2-fold in both IB4-myc23(24/3) and IB4-myc123(24/3) cells when compared to the IB4-HEBo(30/3) control cell line in two out of three experiments but in the other no significant difference was observed (figure 16). Relative increases in expression of CD21 were also observed in both *myc*-transfected cell lines relative to IB4-HEBo(30/3) but significant increases (2-fold) were only seen in one experiment (figure 16). No significant difference in expression of CD23 was observed in IB4-myc123(24/3) cells relative to IB4-HEBo(30/3), but a two-fold increase in expression, relative to the control cell line, was observed in IB4-myc23(24/3).

A set of three further experiments, looking at expression of CD23 in IB4-HEBo(30/3) and IB4-myc23(24/3) cell lines, were performed and results analysed on a FACS flow cytometer (Table 3b, figure 17). On this occasion a highly variable pattern of expression was observed. A mean fluorescence

Table 3

Flow cytometric analysis of B cell surface molecules in IB4 transfectants

Table 3a	IB4-HEBo(30/3)		IB4-myc23(24/3)		IB4-myc123(24/3)	
Antigen	Mean FL/FS	S.E.	Mean FL/FS	S.E.	Mean FL/FS	S.E.
CD10	38.5	7.3	40.5	8.5	43.0	9.4
CD19	64.9	7.4	114.9	19.7	108.6	18.1
CD20	226.2	5.1	220.0	25.4	224.5	30.5
CD21	31.9	5.2	55.6	12.4	75.7	17.8
CD22	38.5	3.2	59.3	14.1	43.4	6.2
CD23	69.1	5.1	189.7	22.6	102.6	11.8
CD30	39.5	2.3	55.1	16.3	56.1	15.8
CDw70	191.5	20.9	141.8	32.9	181.1	29.6
CD71	67.8	8.6	118.1	63.0	75.2	35.1
CD77	39.5	5.6	41.2	5.6	39.1	6.1
Negative	19.4	0.5	18.9	1.3	20.0	2.3

Table 3b	IB4-HEBo(30/3)		IB4-myc23(24/3)	
Antigen	Mean FL/FS	S.E.	Mean FL/FS	S.E.
CD23	1.49	0.54	1.46	0.61
HLA I	4.88	1.32	4.85	0.76
HLA DR	6.94	0.50	6.16	0.48
Negative	0.14	0.003	0.14	0.007

Expression of B cell surface molecules was assessed by indirect immunofluorescence using monoclonal antibodies. Antibody bound to antigen was quantified by incubation with FITC conjugated sheep anti-mouse (or anti-rat in the case of CD77). Staining was analysed on an EPICS PROFILE flow cytometer (Table 3a), where results were expressed as the mean linear fluoresence intensity/size ratio (FL/FS) of 5000 cells; or an EPICS C SYSTEM FACS flow cytometer (Table 3b), where results were expressed as the mean FL/FS of 25000 cells. As a negative control, cells were stained with second layer FITC conjugated antibody alone. Results are the mean and standard errors (S.E.) from three separate experiments. Antibodies used were: CD10, J5; CD19, HD37; CD20, B1; CD21, B2; CD22, TO15; CD23, MHM6; CD30, BerH2; CDw70, Ki24; CD71, BerT9; CD77, 38.13; HLA I, W6.32; HLA DR, L243.

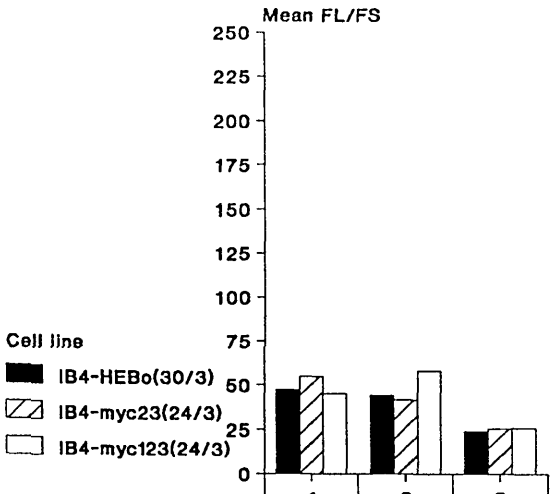
Legend: Figure 16

Flow cytometric analysis of B cell surface antigens.

Expression of B cell surface antigens in IB4-HEBo(30/3), IB4-myc23(24/3) and IB4-myc123(24/3) cell lines was assessed by indirect immunofluorescence using monoclonal antibodies. The mean fluorescence intensity/volume ratio (FL/FS) for 5000 cells was calculated using an EPICS PROFILE flow cytometer. Each histogram represents data from three separate experiments, and the tables below the histograms give the numerical FL/FS values obtained. As a negative control, cells were stained with second layer FITC conjugated anti-mouse IgG alone. Antibodies used were: CD10, J5; CD19, HD37; CD20, B1; CD21, B2; CD22, TO15; CD23, MHM6; CD30, BerH2; CDw70, Ki24; CD71, BerT9 and CD77, 38.13.

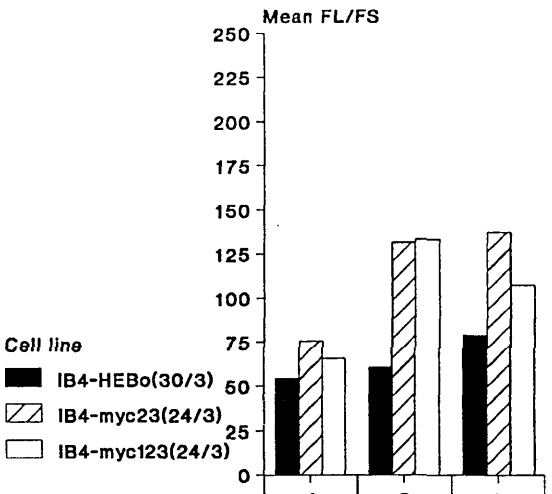
Figure 16

CD10



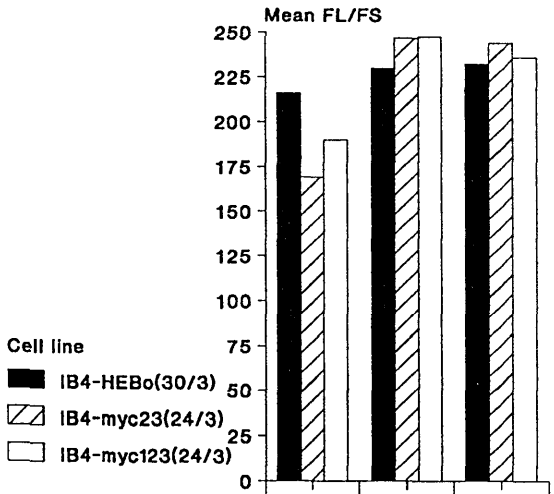
	1	2	3
IB4-HEBo(30/3)	47.2	44.2	24
IB4-myc23(24/3)	54.8	41.4	25.4
IB4-myc123(24/3)	45	58.1	25.9

CD19



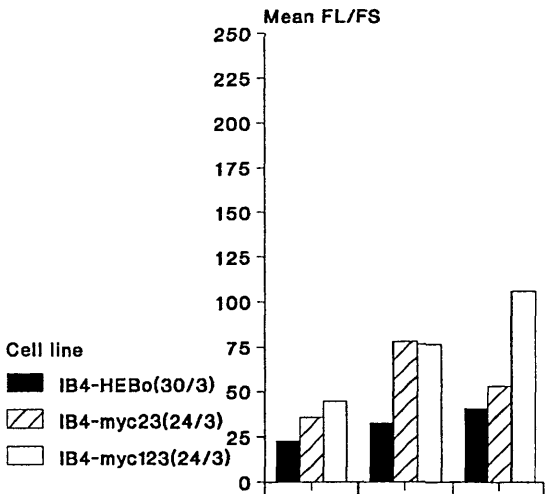
	1	2	3
IB4-HEBo(30/3)	54.5	60.9	79.3
IB4-myc23(24/3)	75.6	131.6	137.5
IB4-myc123(24/3)	65.9	133.4	107.3

CD20



	1	2	3
IB4-HEBo(30/3)	216.1	230	232.4
IB4-myc23(24/3)	169.2	246.7	244
IB4-myc123(24/3)	189.9	247.4	236.1

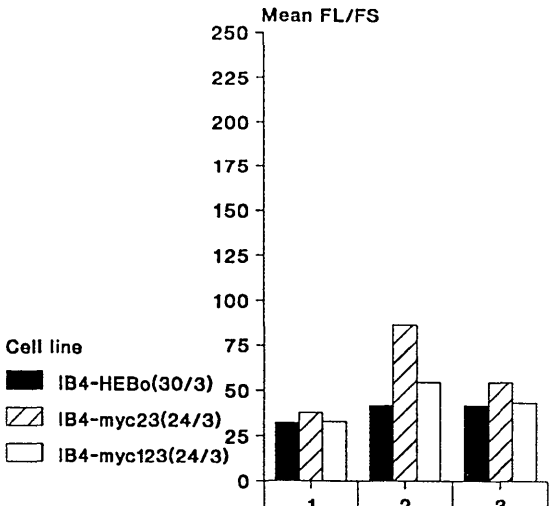
CD21



	1	2	3
IB4-HEBo(30/3)	22.7	32.2	40.7
IB4-myc23(24/3)	35.5	78.3	53.1
IB4-myc123(24/3)	44.4	76.8	106

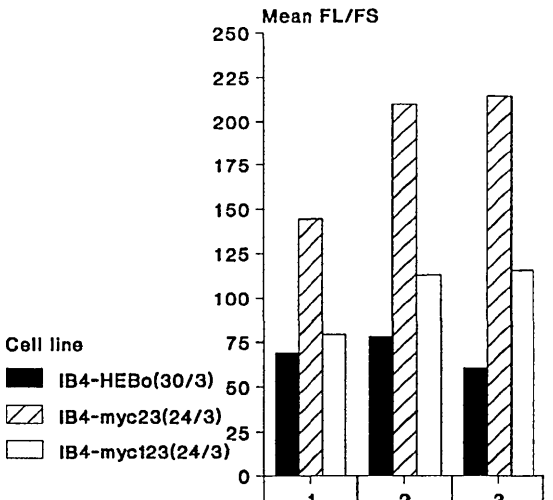
Figure 16 contd.

CD22



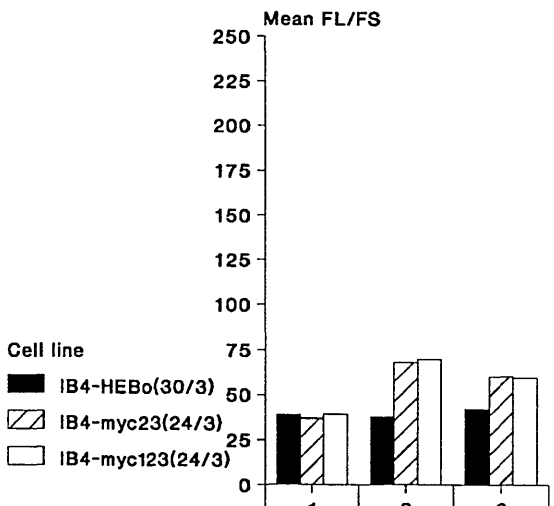
	1	2	3
IB4-HEBo(30/3)	32.2	41.6	41.8
IB4-myc23(24/3)	37.7	86	54.3
IB4-myc123(24/3)	32.8	54.3	43

CD23



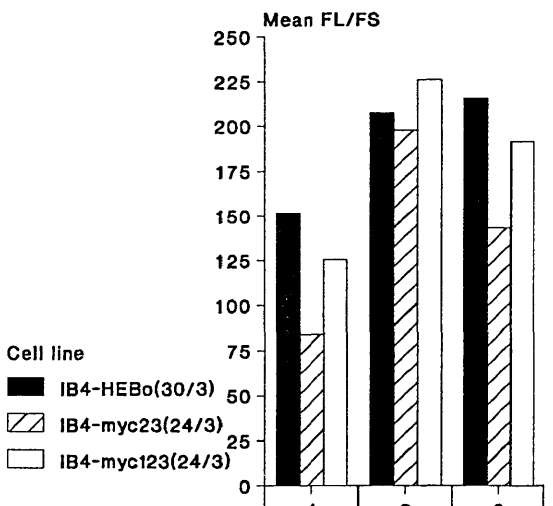
	1	2	3
IB4-HEBo(30/3)	69	78	60.4
IB4-myc23(24/3)	144.5	210	214.5
IB4-myc123(24/3)	79.1	112.9	115.8

CD30



	1	2	3
IB4-HEBo(30/3)	38.8	37.6	42.1
IB4-myc23(24/3)	36.8	68.2	60.3
IB4-myc123(24/3)	39	70	59.4

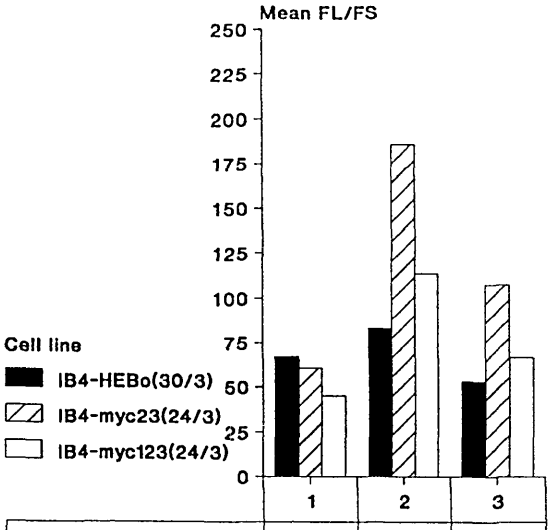
CDw70



	1	2	3
IB4-HEBo(30/3)	151.5	207.4	215.5
IB4-myc23(24/3)	84.1	197.9	143.3
IB4-myc123(24/3)	125.3	226.2	191.7

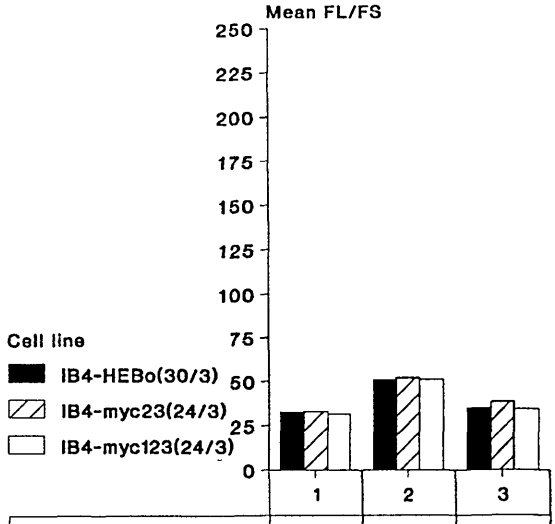
Figure 16 contd.

CD71



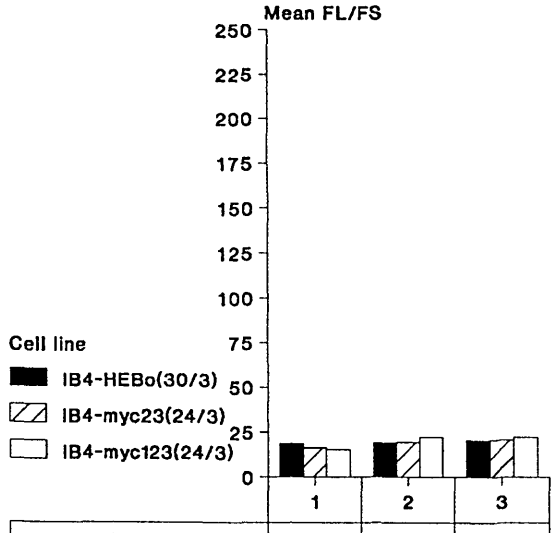
	1	2	3
IB4-HEBo(30/3)	67	83	53.3
IB4-myc23(24/3)	60.8	185.6	107.8
IB4-myc123(24/3)	45.1	113.7	66.8

CD77



	1	2	3
IB4-HEBo(30/3)	32.7	50.7	35
IB4-myc23(24/3)	33	52.1	38.5
IB4-myc123(24/3)	31.6	51.2	34.5

Negative control



	1	2	3
IB4-HEBo(30/3)	18.6	19.2	20.4
IB4-myc23(24/3)	16.4	19.4	20.8
IB4-myc123(24/3)	15.5	22.3	22.2

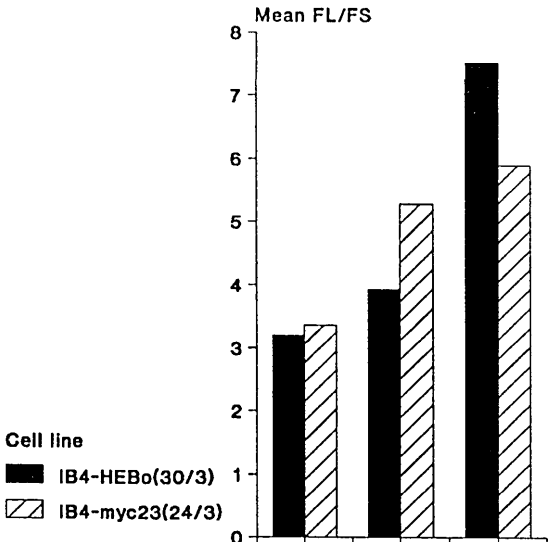
Legend: Figure 17

Flow cytometric analysis of HLA class I, HLA DR and CD23 in IB4-HEBo(30/3) and IB4-myc23(24/3) cell lines.

Expression of HLA class I, HLA DR and CD23 in IB4-HEBo(30/3) and IB4-myc23(24/3) cell lines was assessed by indirect immunofluorescence using monoclonal antibodies. The mean fluorescence intensity of 25000 cells, normalised for cell size, was calculated using an EPICS C SYSTEM FACS flow cytometer. Each histogram represents data from three separate experiments, and table below the histograms give the numerical FL/FS values obtained. As a negative control, cells were stained with second layer FITC-conjugated sheep anti-mouse IgG alone. Antibodies used were: HLA class I, W6.32; HLA DR, L243; CD23, MHM6. *x*-axis, experiment number; *y*-axis, mean linear fluorescence intensity, normalised for size.

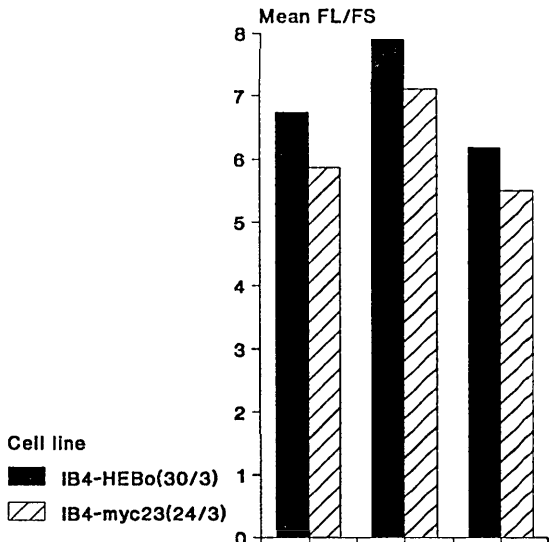
Figure 17

HLA I



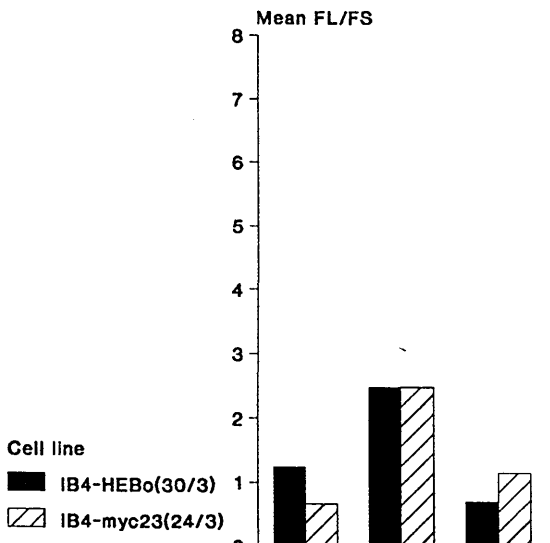
	1	2	3
IB4-HEBo(30/3)	3.2	3.93	7.51
IB4-myc23(24/3)	3.37	5.28	5.9

HLA DR



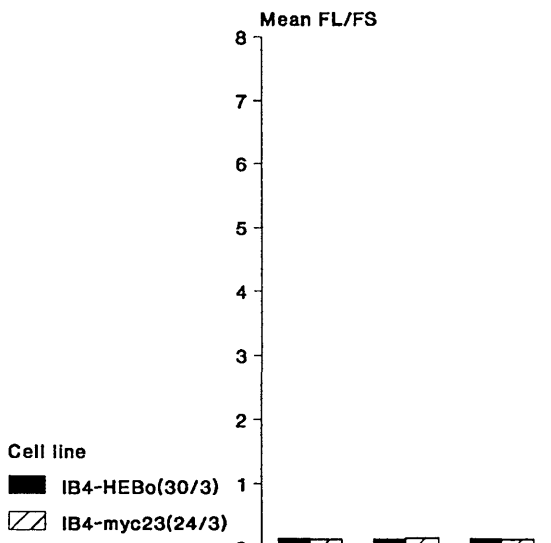
	1	2	3
IB4-HEBo(30/3)	6.74	7.9	6.19
IB4-myc23(24/3)	5.87	7.1	5.51

CD23



	1	2	3
IB4-HEBo(30/3)	1.24	2.48	0.71
IB4-myc23(24/3)	0.67	2.49	1.16

Negative control



	1	2	3
IB4-HEBo(30/3)	0.14	0.13	0.14
IB4-myc23(24/3)	0.13	0.15	0.13

(normalised for size) of 1.49 from the 3 experiments was obtained for IB4-HEBo(30/3) cells compared to 1.46 for IB4-myc23(24/3) cells, indicating no differences in CD23 expression. These values mask the fact that a large variation in CD23 expression between experiments was observed (figure 17, compare experiment 1 & 3). The standard error from the 3 experiments was 0.54 for IB4-HEBo(30/3) and 0.61 for IB4-myc23(24/3).

Expression of CD20, CD22, CD30, CDw70 and CD71 was not significantly changed (Table 3, figure 16). In addition, no significant differences in expression of HLA class I and HLA DR was observed when IB4-HEBo(30/3) and IB4-myc23(24/3) were compared (Table 3b, figure 17).

5.3 Cell adhesion molecules (Table 4).

LFA-1 expression was analysed on the PROFILE flow cytometer using the MHM24 and MHM23 mAbs against the alpha (CD11a) and beta (CD18) subunits respectively. A 2-fold reduction in cell surface expression of CD11a in both the IB4-myc23(24/3) (FL/FS = 73.2) and IB4-myc123(24/3) (FL/FS = 60.5) cell lines, relative to IB4-HEBo(30/3) (FL/FS = 146.3)(Table 4, figure 18), was observed. A similar decrease in expression in CD18 was also observed when IB4-HEBo(30/3) (FL/FS = 133.8), IB4-myc23(24/3) (FL/FS = 71.0) and IB4-myc123(24/3) (FL/FS = 59.5) cell lines were compared. Figure 19 shows a representative profile for the three cell lines stained with MHM24. As can be clearly seen, a marked decrease in fluorescence intensity, and hence cell surface expression, is seen in the *myc*-transfected cell lines. These findings were highly consistent, with standard errors all less than 10%, except for IB4-myc23(24/3) stained with MHM24 (less than 14%).

Expression of CD11a was also assessed using another anti-CD11a mAb (TS1/22) and results analysed on the FACS flow cytometer. Data from 3 separate experiments gave mean fluorescence intensities of 3.03 for CD11a and 2.74 for CD18 in IB4-HEBo(30/3) cells. This compares to values of 1.21 for CD11a and 1.02 for CD18 in IB4-myc23(24/3). So a consistent decrease in expression of LFA-1 of about 2-fold was observed. To confirm that this was not unique to these particular IB4 transfectants, expression of CD11a was examined in the separately established IB4-HEBo(24/3) and IB4-myc23(17/1) cell lines. Comparable decreases in expression of CD11a were observed when IB4-HEBo(24/3) (mean FL = 2.25) was compared to IB4-myc23(24/3) (mean FL = 1.17) (figure 20a) and IB4-myc23(17/1) (mean FL = 1.56) compared to IB4-HEBo(30/3) (mean FL = 2.63) (figure 20b).

Table 4

Flow cytometric analysis of cell adhesion molecule expression in IB4 transfectants

	IB4-HEBo(30/3)		IB4-myc23(24/3)		IB4-myc123(24/3)	
Antigen	Mean FL/FS	S.E.	Mean FL/FS	S.E.	Mean FL/FS	S.E.
CD11a	146.3	5.7	73.2	10.8	60.5	1.6
CD18	133.8	0.6	71.0	6.2	59.5	4.2
CD54	16.0	*	13.8	*	15.0	*
CD58	229.3	8.8	231.3	20.1	233.5	8.5
Negative	19.4	0.5	18.9	1.3	20.0	2.3

* Data from one experiment only.

Expression of cell adhesion molecules was assessed by indirect immunofluorescence using monoclonal antibodies. Intensity of staining was analysed on a PROFILE flow cytometer and expressed as mean linear fluoresence intensity/size ratios of 5000 cells. As a negative control, cells were stained with second layer FITC conjugated sheep anti-mouse antibody alone. Results are the mean and standard error (S.E.) from three separate experiments. Antibodies used were: CD11a, MHM24; CD18, MHM23; CD58, TS2/9; CD54, RR1/1.

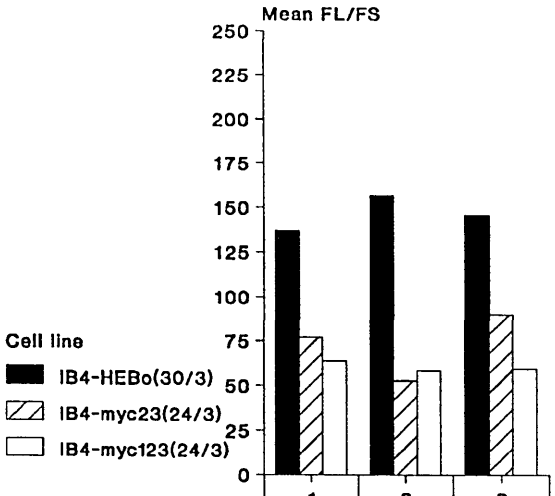
Legend: Figure 18

Flow cytometric analysis of cell adhesion molecule expression in IB4 transfectants.

Expression of LFA-1 alpha subunit (CD11a), LFA-1 beta subunit (CD18) and LFA-3 (CD58) in IB4-HEBo(30/3), IB4-myc23(24/3) and IB4-myc123(24/3) cell lines was assessed by indirect immunofluorescence using monoclonal antibodies. The mean fluorescence intensity/size ratio (FL/FS) for 5000 cells was calculated using an EPICS PROFILE flow cytometer. Each histogram represents data from three separate experiments, and table below the histograms give the numerical FL/FS values obtained. As a negative control, cells were stained with second layer FITC-conjugated sheep anti-mouse IgG alone. Antibodies used were: CD11a, MHM24; CD18, MHM23; CD58, TS2/9. *x*-axis, experiment number; *y*-axis, mean FL/FS.

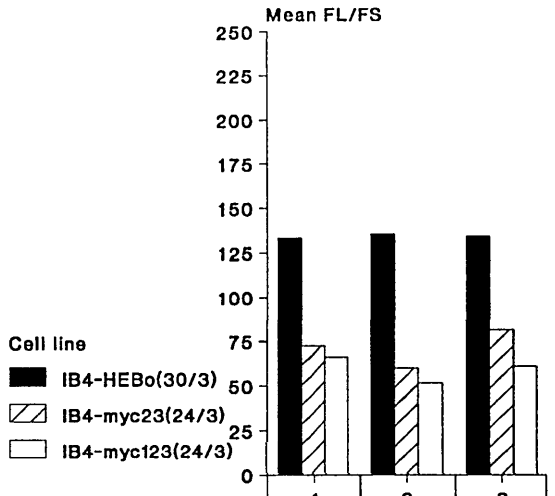
Figure 18

CD11a



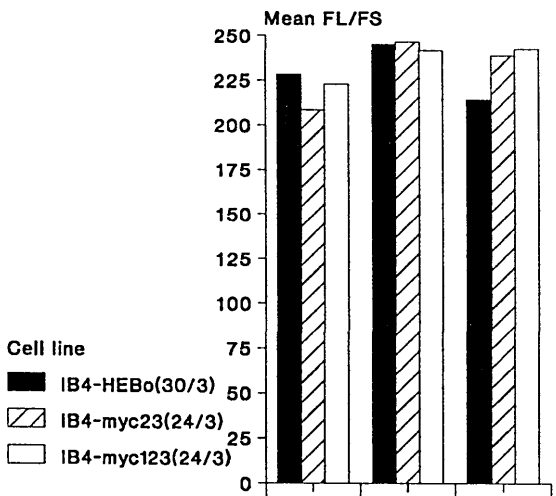
	1	2	3
IB4-HEBo(30/3)	136.7	156.3	145.8
IB4-myc23(24/3)	77.1	52.8	89.9
IB4-myc123(24/3)	63.6	58.4	59.5

CD18



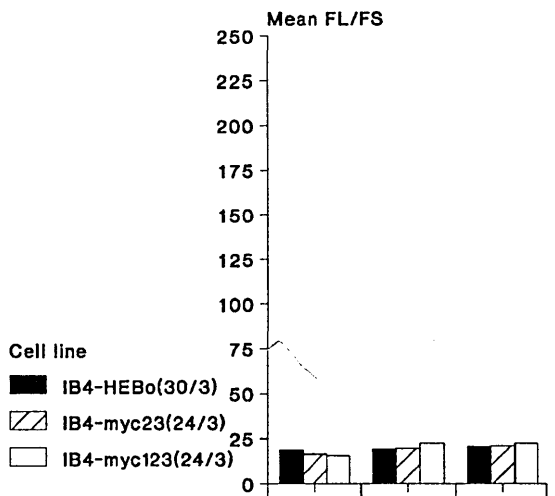
	1	2	3
IB4-HEBo(30/3)	132.7	134.9	133.9
IB4-myc23(24/3)	72.2	59.7	81.2
IB4-myc123(24/3)	66	51.6	60.8

CD58



	1	2	3
IB4-HEBo(30/3)	228.3	244.9	214.2
IB4-myc23(24/3)	208.5	246.5	239
IB4-myc123(24/3)	222.9	241.9	242.4

Negative control

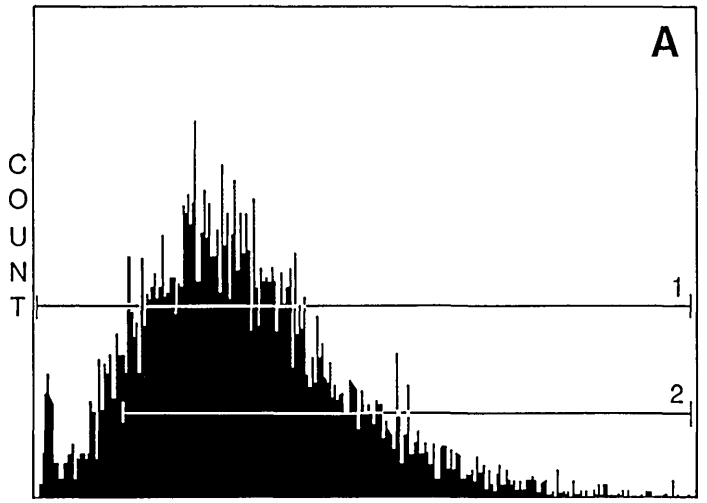


	1	2	3
IB4-HEBo(30/3)	18.6	19.2	20.4
IB4-myc23(24/3)	16.4	19.4	20.8
IB4-myc123(24/3)	15.5	22.3	22.2

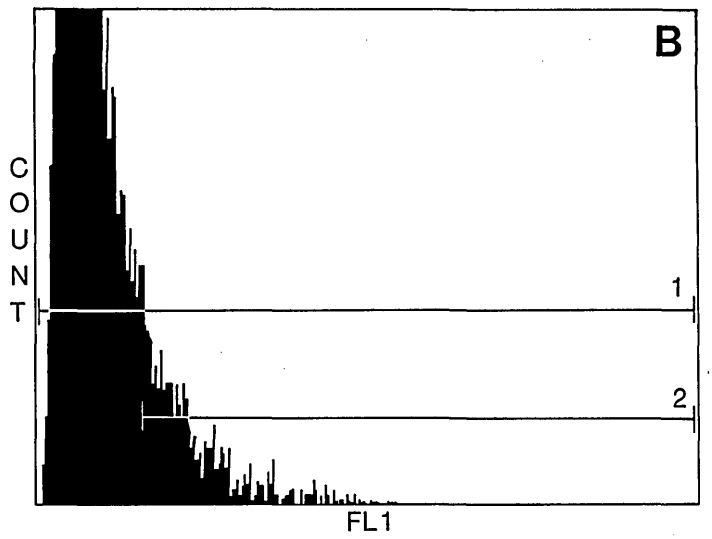
Legend: Figure 19

Graphs showing flow cytometric analysis of CD11a expression in IB4 transfectants

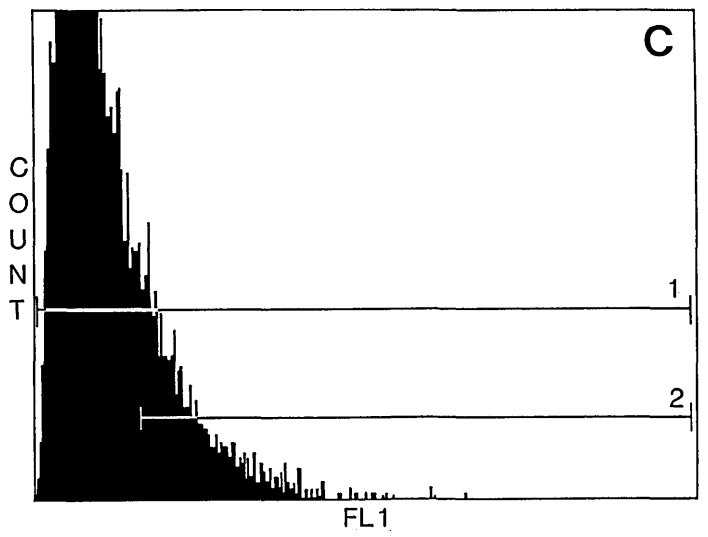
Graphs showing the flow cytometric profiles from a representative experiment analysing expression of CD11a (LFA-1 alpha chain) in IB4-HEBo(30/3) (figure A), IB4-myc23(24/3) (figure B) and IB4-myc123(24/3) (figure C). Cells were stained with an anti-CD11a antibody (MHM24), and analysed on an EPICS PROFILE flow cytometer. The *x*-axis represents linear fluorescence intensity, in terms of channel number (0-256) - the higher the channel number the brighter the fluorescence. The *y*-axis represents number of cells per channel. Cursor 1 is the entire population of cells. Cursor 2 is the population of cells which overlaps with only 1% of the negative control (cells stained with second layer FITC-conjugated sheep anti-mouse IgG alone). The tables below each graph give the numerical values obtained using cursor 1 (1) or cursor 2 (2).



	MIN	MAX	COUNT	PERCENT	MEAN	SD	% HPCV
1	0	255	4999	100.0	80.1	42.9	20.3
2	33	255	4485	89.7	87.1	39.6	20.3



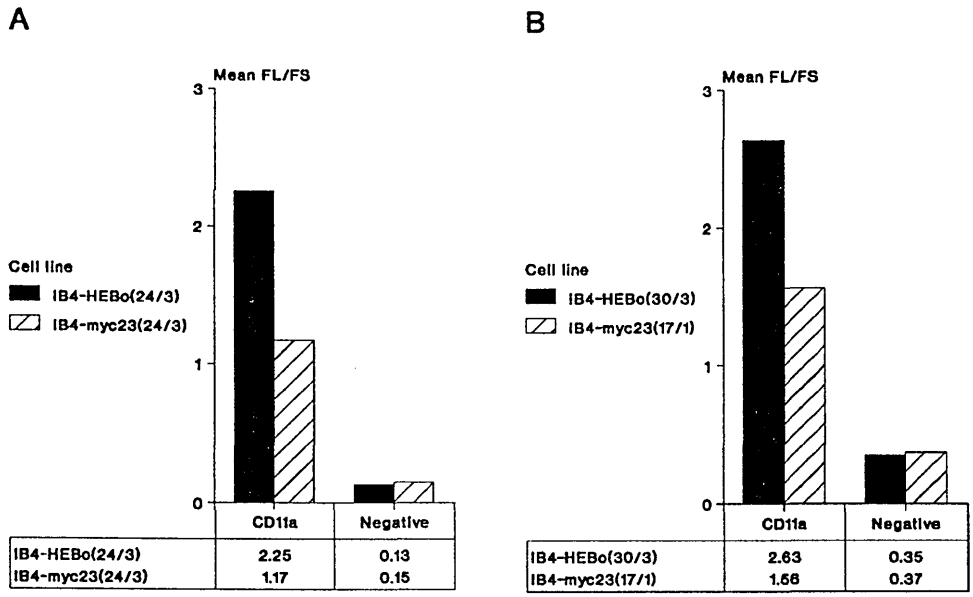
	MIN	MAX	COUNT	PERCENT	MEAN	SD	% HPCV
1	0	255	4999	100.0	23.6	19.0	74.0
2	40	255	723	14.5	59.8	21.9	51.7



	MIN	MAX	COUNT	PERCENT	MEAN	SD	% HPCV
1	0	255	4999	100.0	28.3	24.5	63.2
2	40	255	1096	21.9	64.0	28.2	51.0

Figure 20

Flow cytometric analysis of CD11a expression in separately established IB4 transfectants.



Expression of CD11a (LFA-1 alpha subunit) in IB4-HEBo(24/3), IB4-myc23(24/3), IB4-HEBo(30/3) and IB4-myc23(17/1) cell lines was assessed by indirect immunofluorescence using the TS1/22 monoclonal antibody. Intensity of staining was analysed on a FACS flow cytometer and expressed as mean linear fluoresence intensity of 25000 cells, normalised for cell size (FL/FS). Tables below the histograms are the numerical values for FL/FS. As a negative control, cells were stained with the second layer antibody (FITC-conjugated sheep anti-mouse IgG) alone. Figure A, experiment analysing IB4-HEBo(24/3) and IB4-myc23(24/3); figure B, experiment analysing IB4-HEBo(30/3) and IB4-myc23(17/1)

Expression of LFA-3 (CD58) was analysed using the TS2/9 mAb on the PROFILE flow cytometer. No significant differences in expression between IB4-HEBo(30/3) (FL/FS = 229.3), IB4-myc23(24/3) (FL/FS = 231.3) and IB4-myc123(24/3) (FL/FS = 233.5) was noted (Table 4, figure 18).

Interestingly ICAM-1 (CD54), one of the ligands for LFA-1, was not expressed in either the control or *myc*-transfected IB4 cell lines. FL/FS values for IB4-HEBo(30/3) (16.0), IB4-myc23(24/3) (13.8) and IB4-myc123(24/3) (15.0) were not above levels obtained using second layer sheep anti-mouse FITC conjugate alone (Table 4).

5.4 Expression of LFA-1 in clones of IB4-myc23(24/3)

Expression of CD11a was examined in the seven clones of IB4-myc23(24/3) (section 2.5). Results of staining with the TS1/22 mAb and analysing cells on the FACS flow cytometer are presented in Table 5 and figure 21. Results are the mean FL/FS from 3 separate experiments, except clones 46 and 47 which are from 2 experiments. All 7 of the clones expressed levels of CD11a comparable with the parent IB4-myc23(24/3) cell line (mean FL/FS = 1.21), with no great variation between the 7 clones (mean FL/FS ranging from 1.41 in clone 5 to 0.77 in clone 47). All clones expressed levels of CD11a at least 2 fold less than those observed in IB4-HEBo(30/3) (mean FL = 3.03).

5.5 Summary

In terms of expression of B-cell differentiation antigens both control and *myc*-transfected cell lines possessed the phenotype typical of an LCL, expressing CD21, CD23, CD30 and CDw70. However low level expression of CD10 and CD77, markers of pre-B cells, was also detected suggesting that IB4 cells may represent a relatively early stage of B cell differentiation. Since all cell lines expressed similar levels of these molecules there is no evidence for differentiation status in response to high level expression of c-*myc*.

Changes in expression of CD19, CD21 and CD23 were observed, but these findings were not consistent when repeated. No differences in expression of HLA class I or HLA DR molecules was observed.

A consistent and highly repeatable decrease in expression of LFA-1 on the cell

Table 5

Flow cytometric analysis of CD11a expression in IB4-HEBo(30/3), IB4-myc23(24/3) and 7 clones of IB4-myc23(24/3).

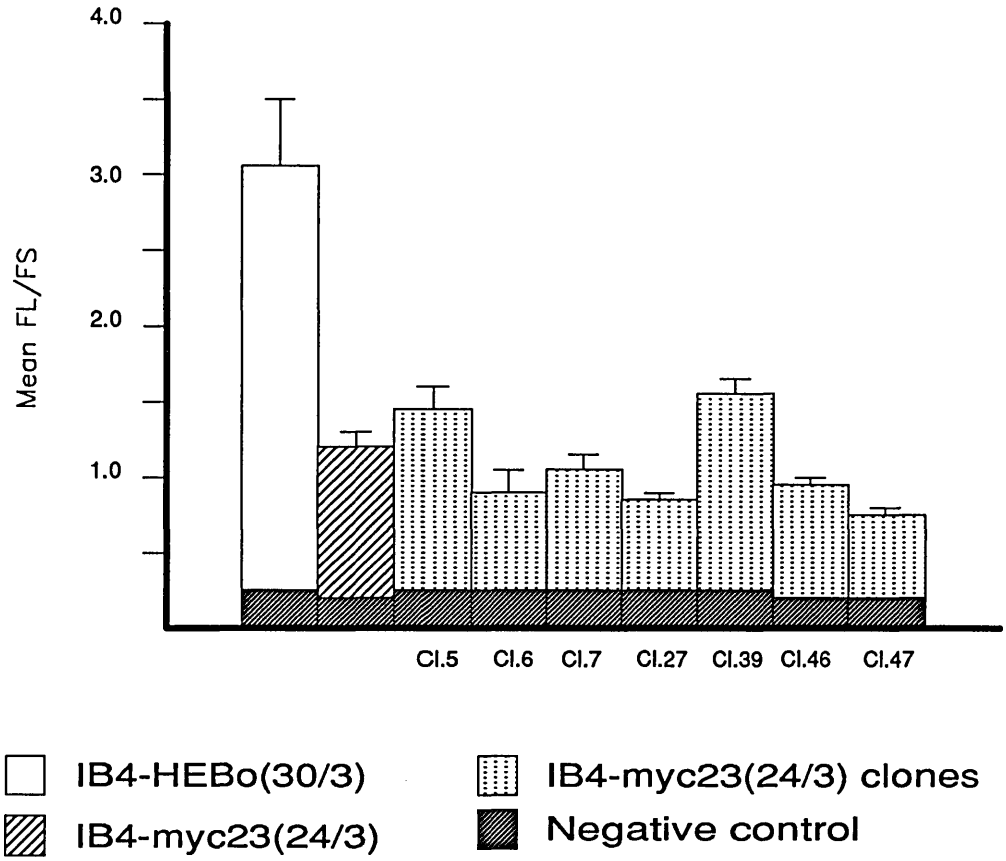
	anti-CD11a		Negative
Cell line	Mean FL/FS	S.E.	Mean FL/FS
IB4-HEBo(30/3)	3.03	0.46	0.24
IB4-myc23(24/3)	1.21	0.11	0.22
IB4-myc23(24/3) clone 5	1.41	0.16	0.25
IB4-myc23(24/3) clone 6	0.89	0.23	0.24
IB4-myc23(24/3) clone 7	1.05	0.23	0.24
IB4-myc23(24/3) clone 27	0.81	0.21	0.26
IB4-myc23(24/3) clone 39	1.27	0.13	0.25
IB4-myc23(24/3) clone 46	0.95*	0.11	0.19
IB4-myc23(24/3) clone 47	0.77*	0.11	0.22

* Mean of 2 experiments only

Expression of CD11a was assessed by indirect immunofluorescence using the TS1/22 monoclonal antibody. Intensity of staining was analysed on a EPICS C SYSTEM FACS flow cytometer and expressed as the mean linear fluoresence intensity of 25000 cells, normalised for mean size (mean FL/FS). As a negative control, cells were stained with the second layer antibody (FITC-conjugated sheep anti-mouse IgG) alone. Results are the mean and standard error (S.E.) of three separate experiments, except for IB4-myc23(24/3) clones 46 and 47 which are the results from two experiments.

Figure 21

Flow cytometric analysis of CD11a expression in clones of IB4-myc23(24/3)



Expression of CD11a (LFA-1 alpha subunit) in the seven clones (Cl.) of IB4-myc23(24/3) was assessed by indirect immunofluorescence using the TS1/22 monoclonal antibody, and compared to IB4-HEBo(30/3) and the parental IB4-myc23(24/3) cell line. Intensity of staining was analysed on an EPICS C SYSTEM FACS flow cytometer and expressed as mean linear fluorescence intensity normalised for cell size (FL/FS) of 25000 cells. As a negative control, cells were stained with the second layer antibody (FITC-conjugated sheep anti-mouse IgG) alone. Histograms represent the mean FL/FS from 3 separate experiments, except for IB4-myc23(24/3) clones 46 and 47, which are the mean of two experiments. Bars indicate the standard error of the mean.

surface was observed in all *myc*-transfected cell lines tested relative to control pHEBoSV-transfected cell lines. This was true for both the alpha and beta subunits of the molecule, which is as might be predicted, since the molecule is expressed on the cell surface as a heterodimer consisting of a single alpha and a single beta subunit (Sanchez-Madrid *et al.*, 1982).

ICAM-1, one of the ligands for LFA-1, was not detected on any of the IB4 cell lines, suggesting that the homotypic B cell adhesion seen in IB4 cells is mediated via interaction of LFA-1 with another ligand, such as the recently described ICAM-2 (Staunton *et al.*, 1989). Antibodies against ICAM-2 are not yet available (D. Staunton, personal communication). Alternatively, there may be an another adhesion pathway involved, however the fact that decreased homotypic cell adhesion was observed in association with decreased expression of LFA-1 in IB4-myc23(24/3) and IB4-myc123(24/3) is strong circumstantial evidence that LFA-1 is involved in homotypic cell adhesion in these cells.

Expression of LFA-3, another molecule important in interactions between EBV infected B cells and cytotoxic T cells, was unchanged when control and *myc*-transfected IB4 cell lines were compared.

6. ANALYSIS OF LFA-1 EXPRESSION

6.1 Introduction

Having observed decreased expression of LFA-1, a molecule of fundamental importance in immune cell interactions, in *myc*-transfected cells the next step was to attempt to determine how expression of this molecule was regulated. Northern blot analysis of mRNA from IB4-HEBo(30/3) and IB4-myc23(24/3) was performed to assess relative levels of the 2 subunit transcripts, and nuclear run-on experiments were carried out to examine relative rates of transcription of the two genes.

6.2 Levels of mRNA

A northern blot of 10μg poly-A selected RNA from IB4-HEBo(30/3) and IB4-myc23(24/3) was sequentially probed, stripped and reprobed with ^{32}P labelled cDNA probes specific for alpha (3R1) and beta (J8) subunit mRNA. DNA probes specific for c-*myc* (pSV-Hu-c-*myc*-1) and the house-keeping gene G6PD (200bp PstI-PstI fragment) were also used (details of probes are given in chapter VI,

section 5). To quantify relative levels of mRNA present in the 2 cell lines the density of hybridisation signal was analysed using a scanning densitometer, and the intensity of signal expressed as a ratio relative to the signal obtained using the control G6PD probe. This was to correct for slightly differing amounts of RNA loaded onto the gel.

As expected, a considerable increase in the amount of c-*myc* mRNA was seen in the *myc*-transfected cell line. Approximately 2 fold less alpha subunit mRNA was detected in the IB4-myc23(24/3) cell line relative to the control IB4-HEBo(30/3) cell line. In contrast, no difference in levels of LFA-1 beta mRNA was observed, indicating that the decreased expression of LFA-1 seen on the cell surface was a consequence of altered LFA-1 alpha subunit gene expression (figure 22).

6.3 Rates of transcription

Nuclear run-on experiments using nuclei prepared from IB4-HEBo(30/3) and IB4-myc23(24/3) were carried out to examine transcriptional activity of LFA-1 alpha and beta subunit genes in IB4-HEBo(30/3) and IB4-myc23(24/3). Nuclei from 10^7 cells of each cell line were isolated and transcription allowed to proceed, *in vitro*, for 30 minutes in the presence of ^{32}P labelled UTP, and unlabelled ATP, GTP and CTP. Incorporation of ^{32}P-UTP (10^7 cpm) was in line with expected values (Dr. D. Bentley Imperial Cancer Research Fund, personal communication). The transcription reactions were hybridised to slot-blotted DNA probes specific for c-*myc* exon II , LFA-1 alpha (3R1), LFA-1 beta (J8) and G6PD (5B) (probes described in chapter VI, section 5). Considerable c-*myc* exon II transcriptional activity was seen in IB4-myc23(24/3) cells relative to IB4-HEBo(30/3) (figure 23). Only very low level hybridisation was observed with the G6PD and LFA-1 specific probes. This was despite exposing the hybridisations to X-ray film for 28 days. However, the results obtained appeared to indicate a decreased rate of LFA-1 alpha subunit transcription in IB4-myc23(24/3) cells, compared to IB4-HEBo(30/3) (figure 23). Rates of LFA-1 beta chain transcription were similar for both IB4-HEBo(30/3) and IB4-myc23(24/3), when hybridisation to the control G6PD DNA was taken into account. Thus it would appear, from these results, that the decreased expression of LFA-1 in *myc*-transfected cells is a consequence of decreased transcription of the LFA-1 alpha chain gene. However, because of the poor quality of the hybridisation signal, it is difficult to draw firm conclusions from these data, and further experiments would be required to confirm these results, and to rule out the possibility of

Legend: Figure 22

Northern blot analysis of RNA from IB4-HEBo(30/3) and IB4-myc23(24/3).

10μg poly A selected RNA from IB4-HEBo(30/3) (lane 1) and IB4-myc23(24/3) (lane 2) was separated on a formaldehyde/agarose gel an transferred to a nylon membrane. The blot was probed with a ^{32}P labelled probe specific for the LFA-1 alpha subunit gene. Following exposure to X-ray film the blot was stripped of hybridised probe and reprobed with a probe specific of the LFA-1 beta subunit gene. This proceedure was repeated, probing for c-*myc* and glucose-6-phosphate dehydrogenase (G6PD) transcripts. To correct for slightly different loading of the two RNA samples, hybridisation of probe to RNA was quantified by analysing the intensity of bands using a scanning densitometer. Ratios, relative to the G6PD signal, were calculated and are indicated under the blots.

Figure 22

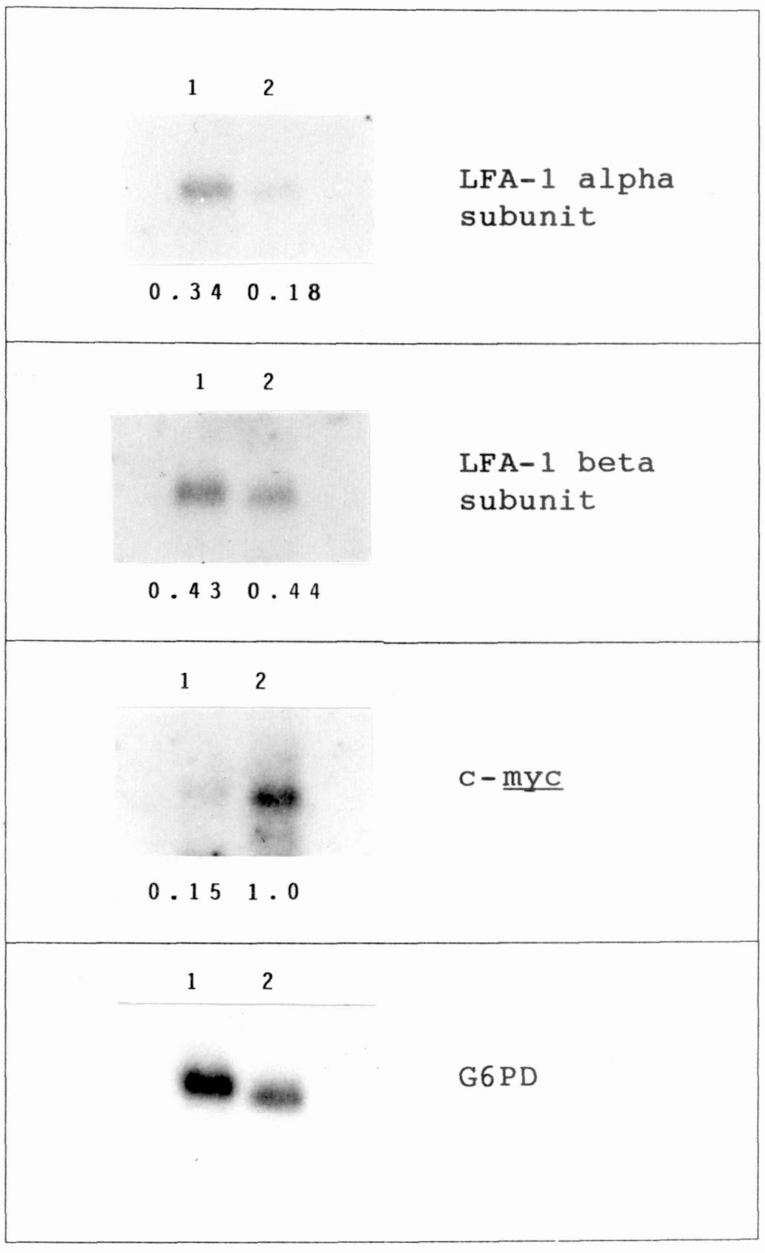
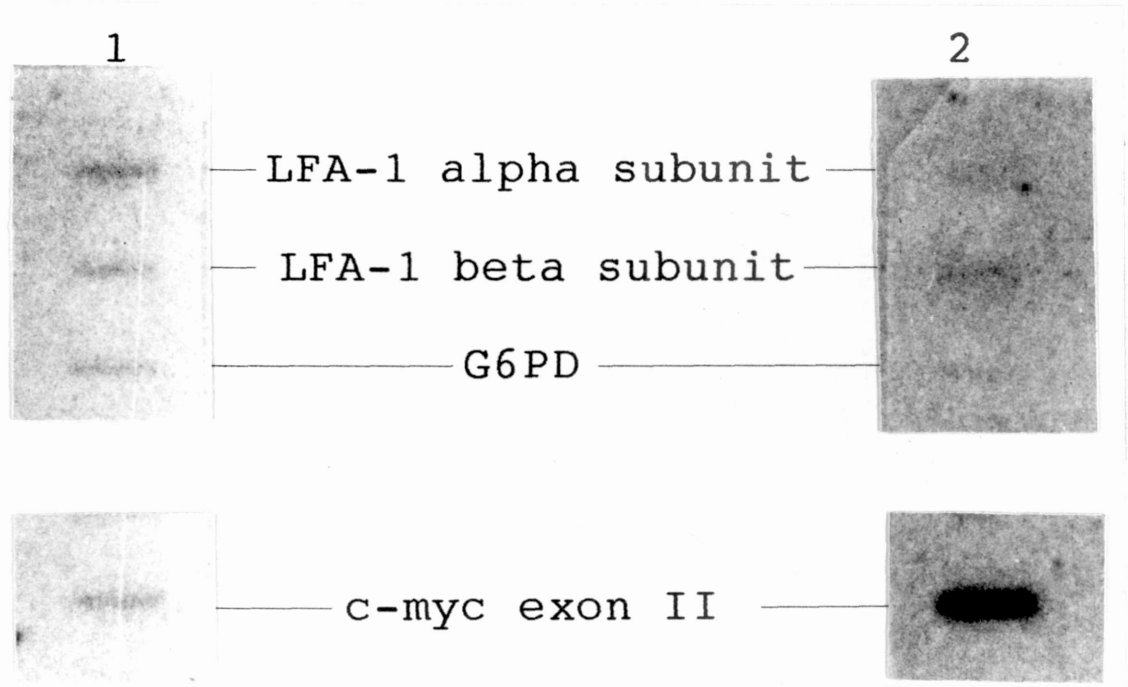

1 2
0.34 0.18
LFA-1 alpha subunit
1 2
0.43 0.44
LFA-1 beta subunit
1 2
0.15 1.0
c-myc
1 2
G6PD

Figure 23

Nuclear run-on analysis of nuclei isolated from IB4-HEBo(30/3) and IB4-myc23(24/3) cell lines.



10^7 nuclei isolated from IB4-HEBo(30/3) and IB4-myc23(24/3) were allowed to transcribe, *in vitro*, for 30 minutes in the presence of ^{32}P labelled UTP. Transcription reactions were hybridised to DNA probes slot-blotted onto nylon membrane. DNA probes specific for LFA-1 alpha and beta gene transcripts, *c-myc* exon II transcripts and G6PD transcripts were used. Lane 1 - IB4-HEBo(30/3); lane 2 - IB4-myc23(24/3).

some post-transcriptional mechanism, such as RNA stability, accounting for the altered expression of LFA-1 alpha subunit.

6.4 Summary

Northern blot analysis revealed reduced levels of LFA-1 alpha subunit mRNA in IB4-myc23(24/3) cells relative to the control cell line, whereas levels of beta subunit transcripts were unchanged. This suggests that the decreased expression of LFA-1 seen at the cell surface is a consequence of altered alpha subunit gene expression. Preliminary nuclear run-on data indicates that a decreased transcriptional activity of the alpha chain gene may be the mechanism regulating the expression of LFA-1. The poor hybridisation signal in the nuclear run-on experiments might be a consequence of the LFA-1 alpha and beta chain genes being transcribed at a very low rate. *De novo* synthesis of the leucocyte integrins is a slow process (Kishimoto *et al.*, 1989) and up-regulation of other members of the leucocyte integrin family on the cell surface is achieved by rapid utilisation of intracellular pools of the alpha and beta subunits rather than increased synthesis of the gene (Miller *et al.*, 1987).

7. EXPRESSION OF LFA-1 IN IB4 CELLS TRANSFECTED WITH MYC MUTANT PLASMIDS

7.1 Introduction

The observation of decreased expression of LFA-1 in cells transfected with c-*myc*, led to the hypothesis that c-*myc* may be acting directly to suppress expression of LFA-1, and that it might be possible to map a region of the c-Myc protein involved in this process. Using a series of deletion mutants Stone *et al* (1987) mapped the regions of protein (residues 106-143, 353-439) essential for the co-transforming activity seen in cooperation with the *ras* oncogene (Land *et al.*, 1983), and for nuclear localization of the protein (residues 320-328, 364-374; Dang and Lee, 1988). Using the same series of mutants Penn *et al* (1990a) have also mapped the regions required for autosuppression, which coincide with those required for co-transformation. To investigate whether the regions involved in autosuppression were in any way involved in suppressing expression of LFA-1, pHEBoSV based plasmid constructions were made containing the same series of mutants for transfection into IB4 cells.

7.2 Construction of pHEBoSVmyc mutants

BamH1-SalI fragments encompassing the mutant c-*myc* genes were isolated

from their parent plasmids (pDORmyc) (figure 24) and ligated into the corresponding sites in pHEBoSV. pHEBoSV was prepared by digesting pHEBoSVmyc23 with BamH1 and SalI and excising the c-*myc* fragment (figure 24). Six plasmids were constructed containing c-*myc* deletions encoding amino acid residues 7-91, 41-178, 145-262, 265-353, 371-412 and 414-433 (Table 6a). Large scale preparations of the plasmids were made and purified on a caesium chloride gradient. Details of digestion, ligation and plasmid preparation techniques are given in chapter VI. The c-*myc* deletion mutants were a gift from Dr W. Lee, University of Philadelphia, USA).

7.3 Transfection of IB4 LCL with mutant plasmids

IB4 cells were transfected with the 6 *myc*-mutant plasmids using electroporation (0.3kV, 960μFD), and 9 stable, hygromycin B resistant cell lines established. At least one cell line was established for each plasmid (Table 6b).

7.4 Expression of c-Myc protein

Immunoblot analysis of c-Myc protein expression in the 9 cell lines transfected with the mutant plasmids, using the myc1-9E10 mAb, revealed no apparent expression of the transfected gene in any of the cell lines (figure 25). The c-Myc protein detected in these cell lines corresponds to endogenous 64kDa protein, with no smaller size proteins visible. Thus, for reasons which are not clear, the partially deleted c-*myc* genes used in this experiment were not expressed when inserted into pHEBoSV and linked to the SV40 early region promoter/enhancer element.

7.5 Expression of LFA-1

In view of the fact that none of the deletion mutants appeared to be expressed, expression of LFA-1 was not analysed.

7.6 Summary

For reasons which are not known, the pHEBoSV based c-*myc* deletion mutant plasmids did not express the deleted c-*myc* genes, despite the fact that hygromycin B resistant cell lines were established, suggesting that the plasmid constructions had been successfully transfected into the cells.

Legend: Figure 24

Construction of pHEBoSV plasmids containing deletion mutants of c-*myc*.

pHEBoSV based plasmid DNA constructions containing c-*myc* Deletion mutants were generated by isolating the mutant genes from pDORmyc and inserting them into pHEBoSV in such a way as to bring the mutant gene under the control of the SV40 promoter/enhancer element. pHEBoSV was prepared by digesting pHEBoSVmyc2,3 with BamH1 and Sal1 restriction endonucleases and isolating, by trough elution, the pHEBoSV fragment of the plasmid. c-*myc* mutant genes were isolated by digesting pDORmyc with the same restriction endonucleases and isolating the mutant gene, which was then ligated into the BamH1-Sal1 digested pHEBoSV plasmid. Using this method six pHEBoSV based plasmids containing c-*myc* genes with deletions covering virtually the entire gene were constructed.

Figure 24

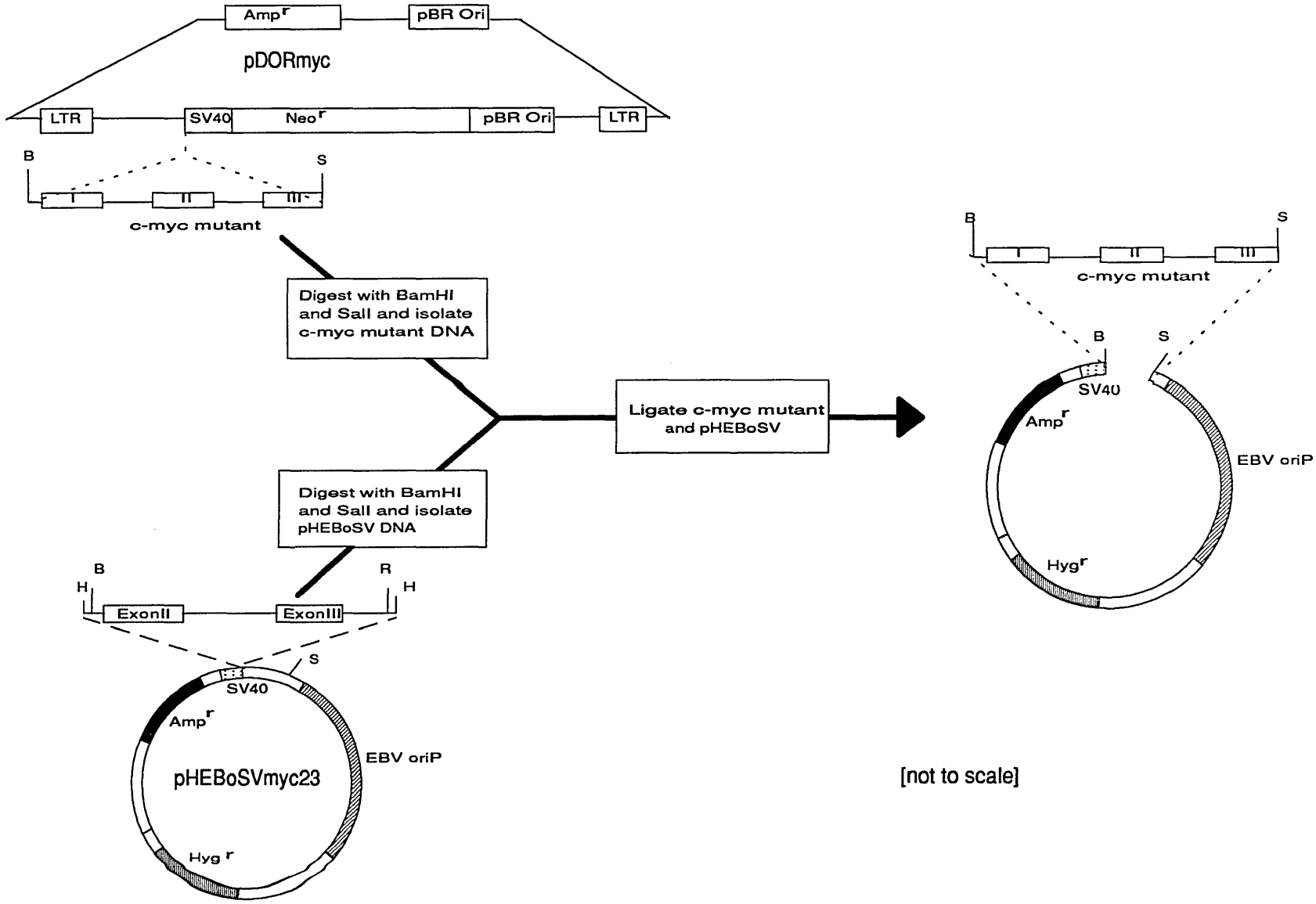

Amp^r
pBR Ori
pDORmyc
LTR
SV40
Neo^r
pBR Ori
LTR
B
S
I
II
III
c-myc mutant
Digest with BamHI and SalI and isolate c-myc mutant DNA
Ligate c-myc mutant and pHEBoSV
Digest with BamHI and SalI and isolate pHEBoSV DNA
B
H
R
H
ExonII
ExonIII
S
SV40
Amp^r
pHEBoSVmyc23
EBV oriP
Hyg^r
B
S
I
II
III
c-myc mutant
B
S
SV40
Amp^r
EBV oriP
Hyg^r
[not to scale]

Table 6

pHEBoSV plasmid constructions containing c-*myc* deletion mutants and cell lines established using these plasmids

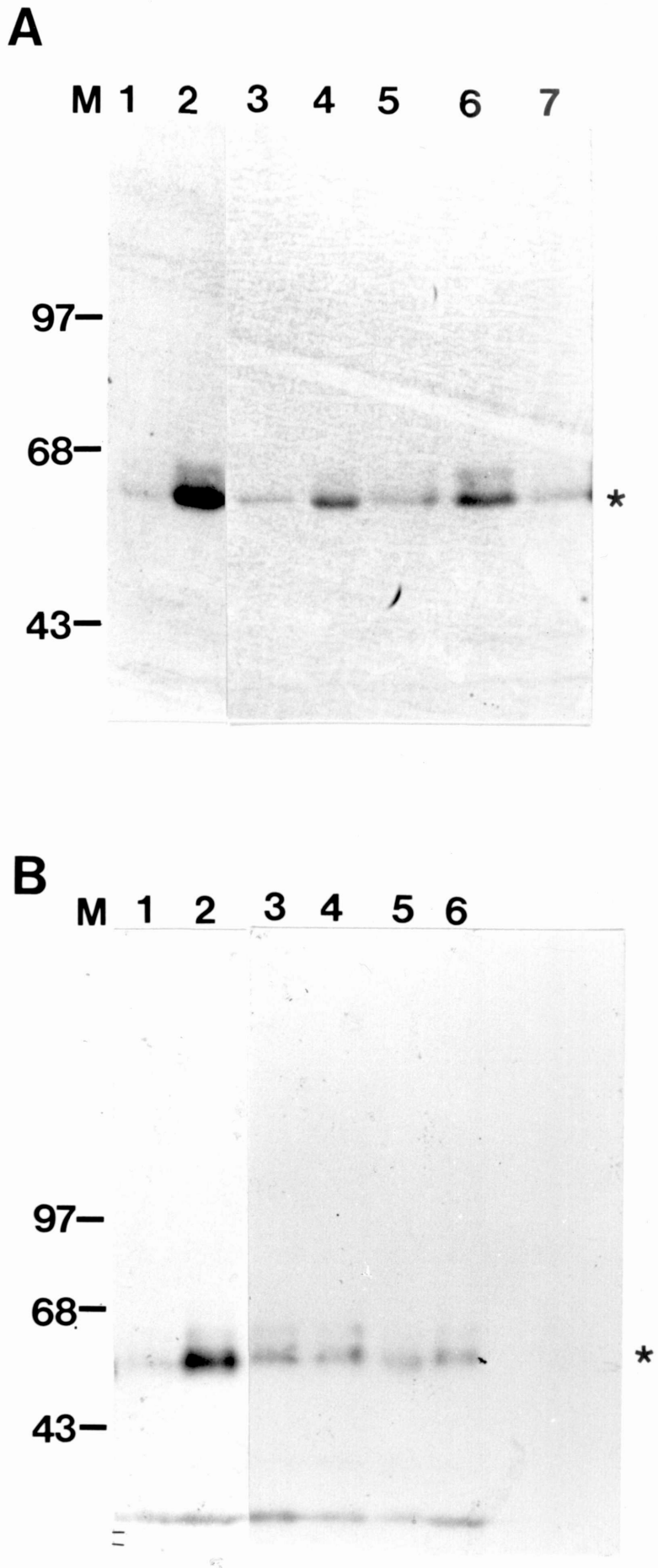
Plasmid constructions
pHEBoSVmyc.del.7-91
pHEBoSVmyc.del.41-178
pHEBoSVmyc.del.145-262
pHEBoSVmyc.del.265-353
pHEBoSVmyc.del.371-412
pHEBoSVmyc.del.414-433

Cell lines
IB4-myc.del.7-91(30/3)
IB4-myc.del.41-178(22/3)
IB4-myc.del.145-262(12/3)
IB4-myc.del.145-262(22/3)
IB4-myc.del.265-353(9/2)
IB4-myc.del.265-353(12/3)
IB4-myc.del.265-353(22/3)
IB4-myc.del.371-412(9/2)
IB4-myc.del.414-433(9/2)

Legend: Figure 25

Immunoblot analysis of c-Myc expression in IB4 cells transfected with c-*myc* deletion mutant plasmids

Protein extracted from 2 x 10^6 cells was separated on a 10% polyacrylamide gel in the presence of 0.1% SDS, transferred to nitrocellulose and probed with a c-Myc specific monoclonal antibody (Myc1-9E10). A: track 1, IB4-HEBo(30/3); track 2, IB4-myc123(24/3); track 3, IB4-myc.del.7-91(30/3); track 4, IB4-myc.del.41-178 (22/3); track 5, IB4-myc.del.145-262(12/3); track 6, IB4-myc.del.145-262(22/3); track 7, IB4-myc.del.265-353(265-353). B: track 1, IB4-HEBo(30/3); track 2, IB4-myc123(24/3); track 3, IB4-myc.del.265-353(12/3); track 4, IB4-myc.del.265-353(22/3); track 5, IB4-myc.del.371-412(9/2); track 6, IB4-myc.del.414-433(9/2). Positions of molecular weight markers (M) and c-Myc proteins corresponding to the normal sizes of 64 and 67kDa (*) are indicated in both figures.


A
M 1 2 3 4 5 6 7
97–
68–
43–
*
B
M 1 2 3 4 5 6
97–
68–
43–
*

8. EBV GENE EXPRESSION

8.1 Introduction

Burkitt's lymphoma cells have a very restricted pattern of EBV gene expression with only one of the latency associated antigens, EBNA-1, being expressed (Rowe D., *et al.*, 1986; Rowe M., *et al.*, 1987b). LCLs by contrast, express most if not all of the identified nuclear proteins (EBNA 1-6), and the latent membrane protein (LMP) (Rowe D., *et al.*, 1986; Ricksten *et al.*, 1988; Allday *et al.*, 1988). Expression of the lytic cycle antigens, VCA and EA, is more variable with some LCL expressing VCA and EA and others apparently not (Rowe *et al.*, 1986). In most LCLs a small number of cells enter the lytic cycle and express VCA and EA. In general it appears that LCLs derived by infecting adult B cells express higher levels than those derived by infecting cord blood lymphocytes (Crawford *et al.*, 1979). Increased expression of lytic cycle antigens, in some LCL and BL cell lines, can be induced by treating with the phorbol ester 12-O-tetradecanoyl-phorbol-1,3-acetate (TPA) (Zur Hausen *et al.*, 1978). TPA has been shown to induce differentiation of B cells (Totterman *et al.*, 1980), and Crawford and Ando (1986) suggested that lytic cycle antigens are only expressed in those cells which are undergoing terminal differentiation.

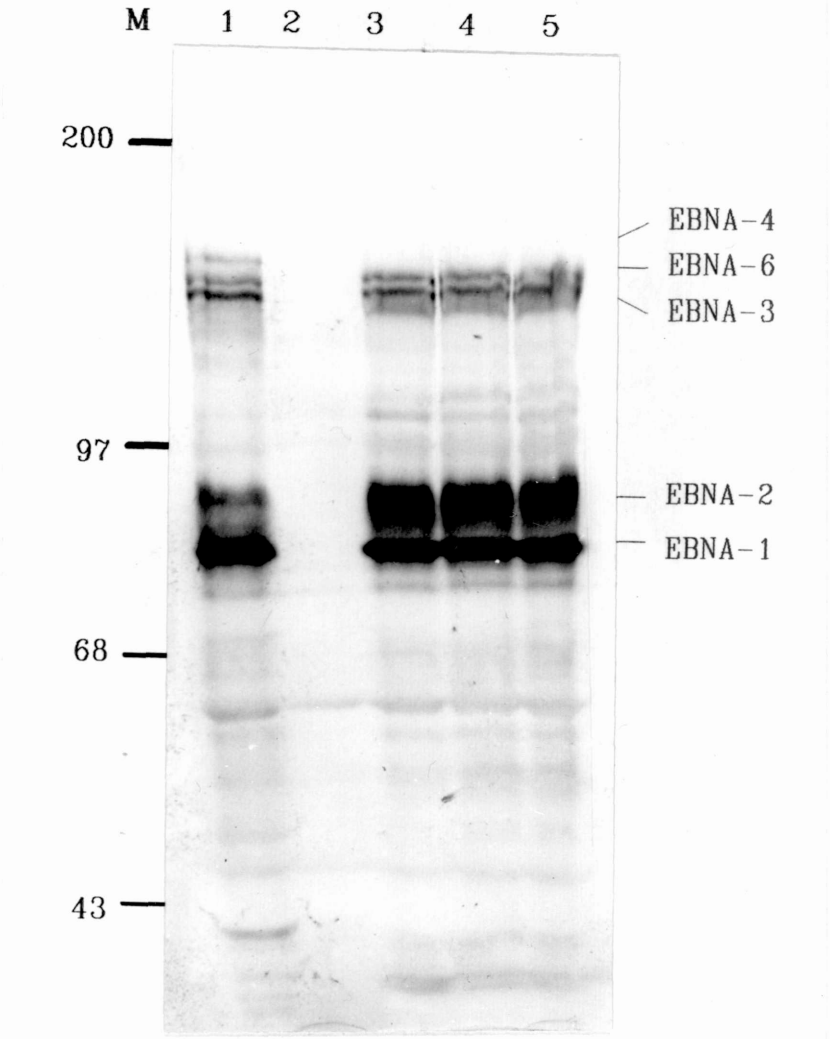
The aim of this set of experiments was two-fold. Firstly, expression of the latency associated and lytic cycle antigens was analysed in *myc*-transfected cell lines and compared to the control cell line. Secondly, experiments were performed to see whether expression of lytic cycle antigens could be induced in IB4 cells with TPA, and whether constitutive expression of c-*myc* affected this induction.

8.2 EBNA expression

Expression of EBNAs in IB4 transfectants was examined by immunoblotting using a human polyclonal serum RT, which recognises all the EBNAs with the exception of EBNA-5 (Allday *et al.*, 1988), and a mouse mAb JF186 (Finke *et al.*, 1987) to detect EBNA-5. Figure 26 shows the results of using RT serum to probe for the presence of EBNA-1, 2, 3, 4 and 6. As can be seen no difference in expression of EBNA 1, 2, 3 and 6 was noted when IB4-HEBo(30/3), IB4-myc23(24/3) and IB4-myc123(24/3) cell lines were compared. EBNA-4 is expressed in the B95-8, the cell line from which the virus used to establish the IB4 cell line was obtained (King *et al.*, 1980), but not in the parental non-transfected IB4 LCL (Rowe *et al.*, 1988; Allday *et al.*, 1988). Similarly EBNA-4

Figure 26

Immunoblot analysis of EBV nuclear antigens in IB4 transfectants.



Protein extracted from 2 x 10^6 cells of the B95-8 (lane 1), Ramos (EBV negative, lane 2), IB4-HEBo(30/3) (lane 3), IB4-myc23(24/3) (lane 4) and IB4-myc123(24/3) (lane 5) cell lines, separated on an SDS-PAGE gel, transferred to nitrocellulose and probed with mouse monoclonal antibodies and human polyclonal serum. Detection of EBNAs 1, 2, 3, 4 and 6, using RT serum. High molecular weight standards were run as markers (lane M).

was not detected in either control or *myc*-transfected IB4 cells. EBNA5 was detected in B95-8 and all of the IB4 transfectants, with no apparent difference in expression (figure 27a). None of the EBNAs were detected in the control EBV-negative BL cell line Ramos.

8.3 LMP expression

Expression of LMP was analysed by immunoblotting using a pool of mAbs (CS1-4) (Rowe *et al.*, 1987). IB4 cells express LMP (Rowe *et al.*, 1988) and all of the IB4 transfectants were similarly LMP positive, with no detectable difference in levels of expression observed (figure 27b). LMP was not present in the EBV negative control Ramos.

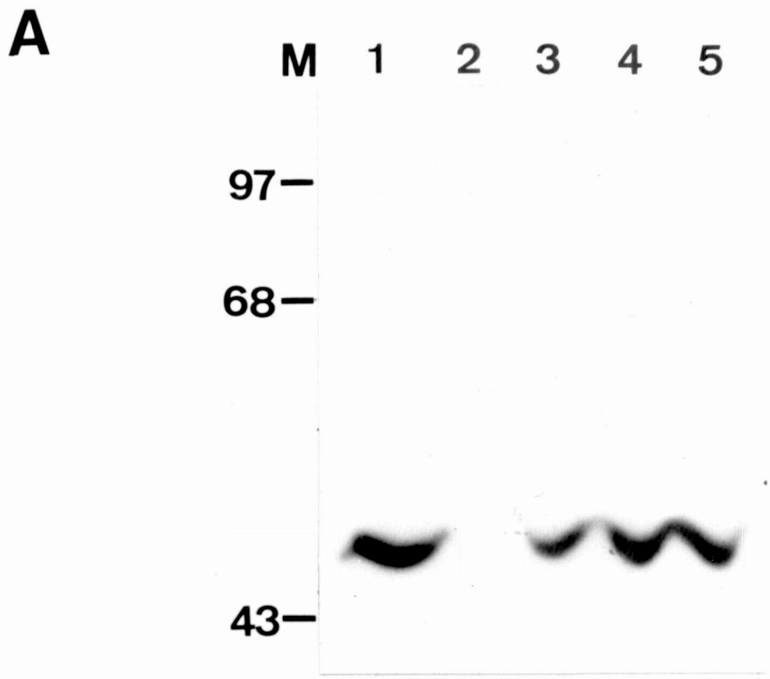
When expression of LMP in IB4-HEBo(30/3) and IB4-myc123(24/3) cells was examined by indirect IF using the CS1-4 antibody an unusual pattern of staining was visible in the *myc*-transfected cell line (figure 28). Instead of the evenly distributed, punctate staining seen in the control cell line, LMP appeared to be localised to discrete patches of intense staining within the cytoplasm. This result is not consistent with the findings of Liebowitz and colleagues (Liebowitz, *et al.*, 1986, 1987) who described co-localisation of LMP and vimentin to patches at the periphery of both LCL and BL cells, including cells from the IB4 LCL. The localisation of LMP to patches in the periphery of lymphoblastoid cells described by Liebowitz and colleagues has not been observed in our laboratory, and a more diffuse pattern of staining, similar to that seen in IB4-HEBo(30/3) is normally observed in LCLs (D.H. Crawford, unpublished data). Whether these differences reflect differing staining techniques is not known. It does not reflect differing antibody specificity since the LMP antibody used by Liebowitz *et al* (S12) show a similar diffuse staining pattern in LCL (D.H. Crawford, unpublished data).

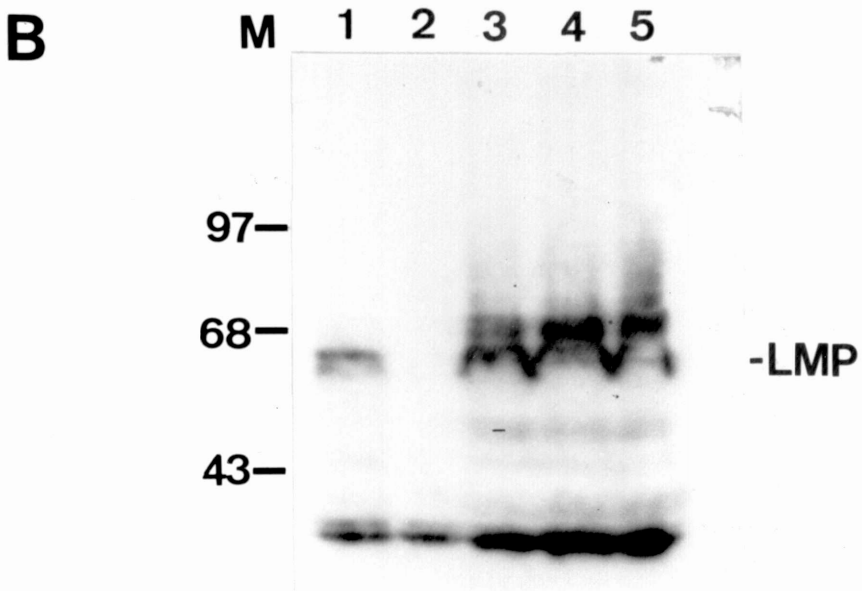
Because of the reported association of LMP with vimentin intermediate filaments (Liebowitz *et al.*, 1987; Birkenbach *et al.*, 1989), expression of vimentin was examined by indirect IF and immunoblotting, using a mAb (V9, Dako). Results of indirect IF on IB4-HEBo(30/3), IB4-myc123(24/3) and Raji cells are shown in figure 29a. Staining for vimentin was doubled with ACIF staining for EBNA using a human polyclonal EBV positive serum (JAT), to act as a counterstain. As can be clearly seen the two IB4-myc123(24/3) cells expressed appreciably less vimentin than the control pHEBoSV-transfected cell line. Some expression of vimentin was detected in Raji cells, which had been previously reported as not expressing vimentin (Dellagi *et al.*, 1984). Vimentin

Legend: Figure 27

Detection of EBNA-5 and LMP in IB4 transfectants by immunoblotting

Immunoblot analysis of EBNA-5 and LMP expression in IB4-HEBo(30/3), IB4-myc23(24/3) and IB4-myc123(24/3). Protein extracted from 2 x 10^6 cells was separated on an SDS-PAGE gel, transferred to nitrocellulose by western blotting and probed for A: EBNA-5 using JF186 anti-EBNA-5 mouse monoclonal antibody, and B: LMP, using a pool of mouse monoclonal antibodies (CS1-4). As controls B95-8 (EBV positive) and Ramos (EBV negative) cell lines were included. Lane 1, B95-8; lane 2, Ramos; lane 3, IB4-HEBo(30/3); lane 4, IB4-myc23(24/3); lane 5, IB4-myc123(24/3). Positions of high molecular weight standards are indicated (M).


A
M 1 2 3 4 5
97
68
43

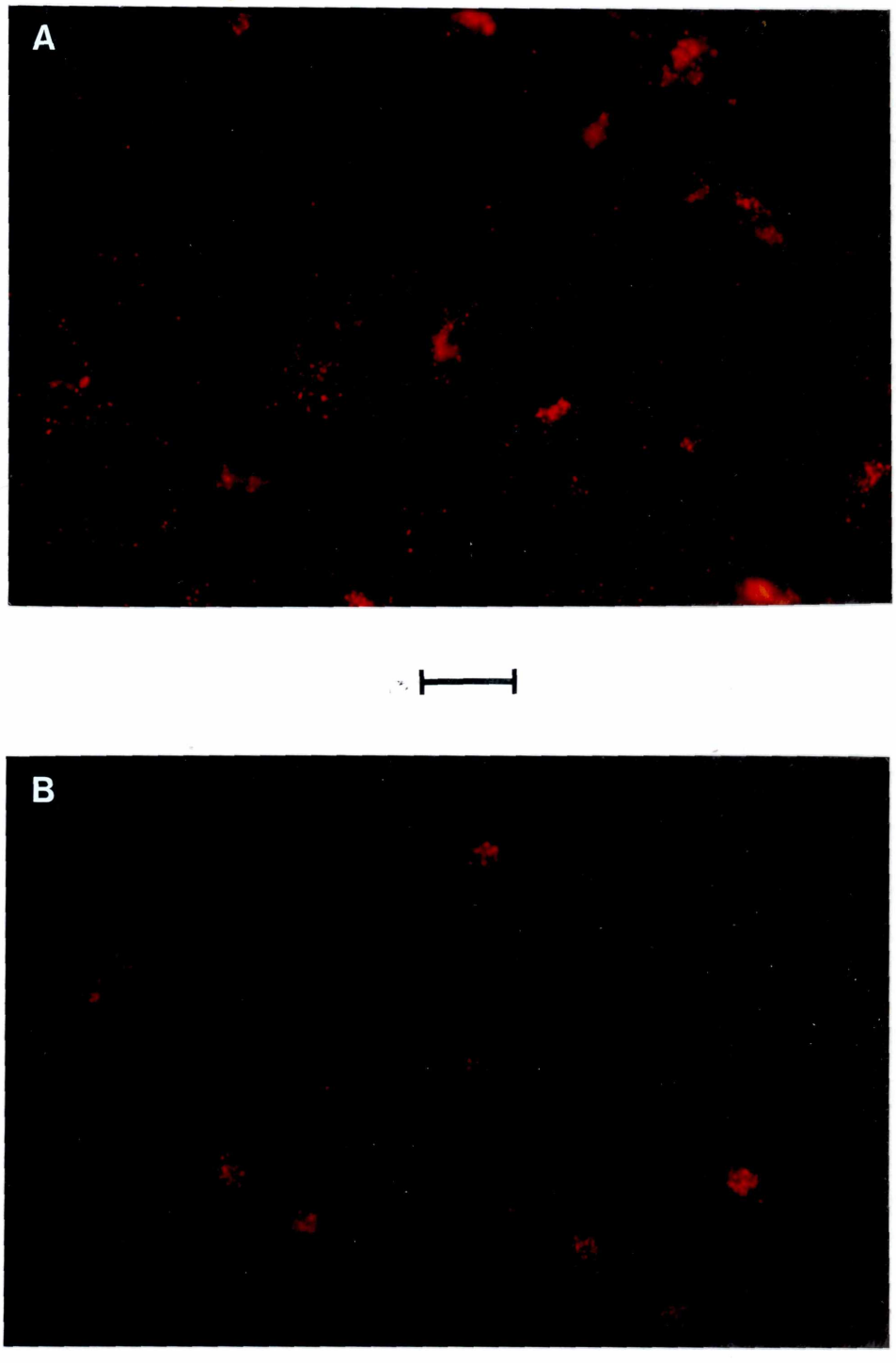

B
M 1 2 3 4 5
97
68
43
-LMP

Legend: Figure 28

Photomicrographs showing expression of LMP in IB4 transfectants

Expression of LMP in IB4-HEBo(30/3) (figure A) and IB4-myc123(24/3) (figure B) was examined by indirect immunofluorescence using a first layer anti-LMP mouse monoclonal antibody pool (CS1-4), and a second layer TRITC conjugated goat anti-mouse IgG. Cells were photographed on a U/V microscope at x1000 magnification. Scale bar represents 10mm.

Figure 28



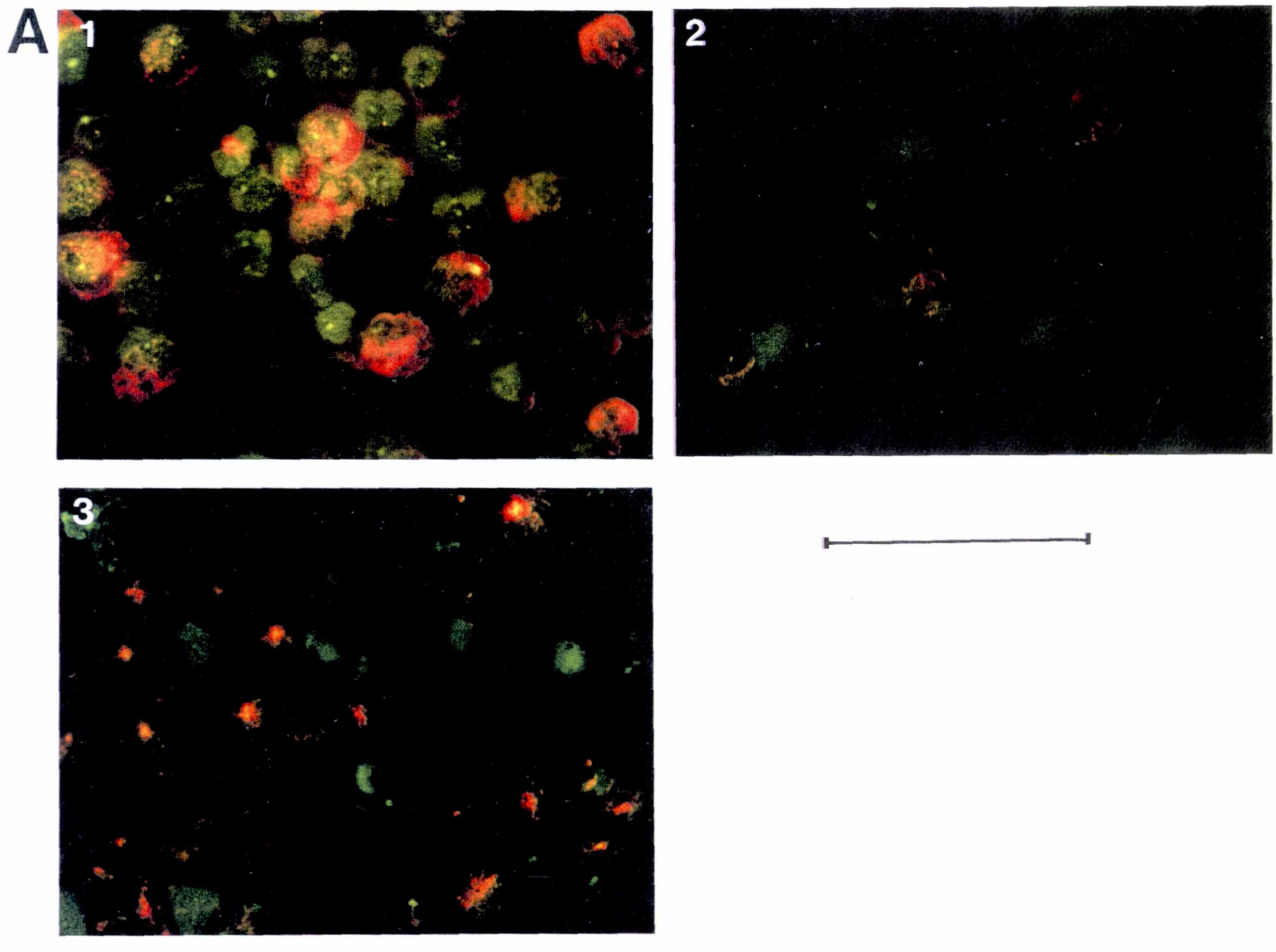
Legend: Figure 29

Detection of vimentin by indirect immunofluorescence and immunoblotting.

A. Expression of vimentin and EBNA in IB4-HEBo(30/3) (figure 1), IB4-myc123(24/3) (figure 2) and Raji (figure 3) cells was assessed by using indirect immunofluorescence to detect vimentin and anti-complement immunofluorescence to detect EBNA. As a first layer, a mixture of human EBNA positive serum (JAT) and a mouse monoclonal antibody (V9) against vimentin containing 10% human complement was added. Staining was differentiated by using, as a second layer, a mixture of TRITC-conjugated goat anti-mouse IgG (to detect vimentin) and FITC-conjugated goat anti-human C3c (to detect EBNA). Cells were photographed on a U/V microscope at x400 magnification (vimentin - red fluorescence, EBNA - green fluorescence). Scale bar represents 50μm.

B. Protein, extracted from 10^6 cells, was separated on a SDS-PAGE gel (10% acrylamide) and transferred to nitrocellulose by western blotting. Vimentin expression was analysed by immunoblotting with a mouse monoclonal antibody (V9) against human vimentin. Lane 1, IB4 LCL; lane 2, IB4-HEBo(12/3); lane 3, IB4-HEBo(30/3); lane 4, IB4-myc23(24/3); lane 5, IB4-myc123(24/3); lane 6, Raji (BL cell line); lane 7, ELI (BL cell line); lane 8, Namalwa (BL cell line); lane 9, Ramos (EBV negative BL cell line); lane 10, PS LCL. Positions of high molecular weight protein standards are shown (M).

Figure 29



in Raji cells, however, was not organised into filaments, but was localised to discrete patches apparently on or near the cell membrane. Immunoblotting of these cell lines with the same antibody revealed similar differences, with very little expression of vimentin detected in IB4-myc23(24/3) and IB4-myc123(24/3) cells (figure 29b). No vimentin was detected in ELI or Namalwa, both EBV positive BL cell lines, or in Ramos an EBV negative BL cell line. Low levels of vimentin were detected in Raji cells. High levels of vimentin were detected in the parental IB4 cell line, and also in another LCL (PS).

8.4 VCA and EA expression

Expression of VCA and EA was determined by indirect IF on acetone fixed cells using polyclonal human sera [Bull (VCA+, EA+); JAT (VCA+, EA-); WDA (VCA-, EA-)]. The virus producing B95-8 cell line was used as a positive control for VCA, and Raji cells treated with TPA (20ng/ml) as a control for EA. Raji cells are unusual in that they express EA but not VCA after treatment with TPA (Henle W., *et al.*, 1970; Zur Hausen *et al.*, 1978). Data presented here are the mean of three separate experiments unless stated. For each experiment slides were prepared by cytospinning 10^5 cells, fixing, staining with the relevant antibodies and the number of positive cells counted. It was assumed that all of the 10^5 cells were applied to the slide by the cytospin.

Very low level expression of EA was detected in IB4 (0.004% of stained cells) and IB4-HEBo(30/3) (0.001%). No expression of EA was detected in IB4-myc123(24/3) (figure 30, Table 7). EA was detected in 0.001% of uninduced Raji cells and 0.05% of TPA treated cells. VCA was not detected in any IB4 transfectant or the parental IB4 cell line, but was detected in the control B95-8 cell line (figure 30).

In parallel experiments, cells were treated with TPA (20ng/ml) and stained by indirect IF for VCA and EA after 72 hours. Expression of EA was detected in 0.03% of TPA-treated IB4 and 0.046% of TPA treated IB4-HEBo(30/3) cells. Expression of EA was detected in TPA-treated IB4-myc123(24/3) cells but only in 0.013% of cells (Table 7, figure 31). Expression of EA was also detected in 0.05% of control, TPA treated, Raji cells. VCA was detected in control TPA treated B95-8 cells, but not in any of the TPA treated IB4 cell lines.

A single experiment, using the same experimental procedure, was also performed using the IB4-HEBo(12/3) and IB4-myc23(24/3) cell lines. In the control cell line EA was expressed in 0.002% of untreated cells and 0.095% of

Table 7

Expression of Epstein-Barr virus early antigen in TPA treated and untreated cell lines

	Untreated		Treated with TPA	
Cell line	No. EA positive	% EA positive	No. EA positive	%EA positive
IB4	4	0.004	30	0.03
IB4-HEBo(12/3)*	2	0.002	95	0.095
IB4-HEBo(30/3)	2	0.002	46	0.046
IB4-myc23(24/3)*	0	0	0	0
IB4-myc123(24/3)	0	0	13	0.013
Raji	1	0.001	50	0.05

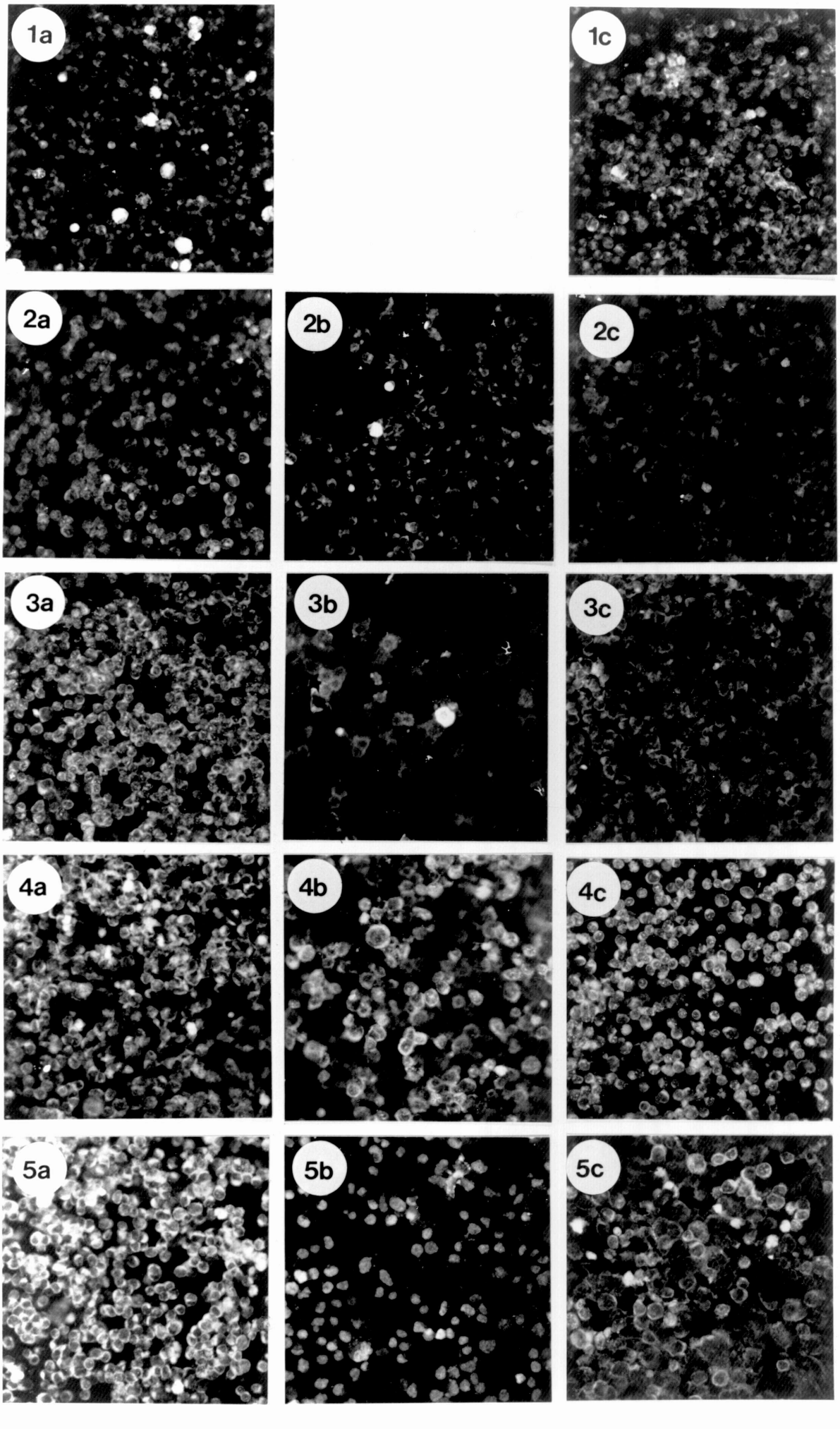
* Data from one experiment only

Table showing proportion of cells expressing the EBV lytic cycle early antigen complex (EA). Expression of EA was determined by indirect immunofluorescence using a human polyclonal serum (Bull). Parallel cultures of TPA treated and untreated cells were stained after 72 hours in culture. Staining was carried out on acetone-fixed cytospin preparations made from 10^5 cells. For the purposes of calculating the percentage of positive cells it was assumed that all 10^5 cells were applied to the glass slides by the cytospin procedure.

Legend: Figure 30

Expression of EBV lytic cycle antigens in IB4 transfectants

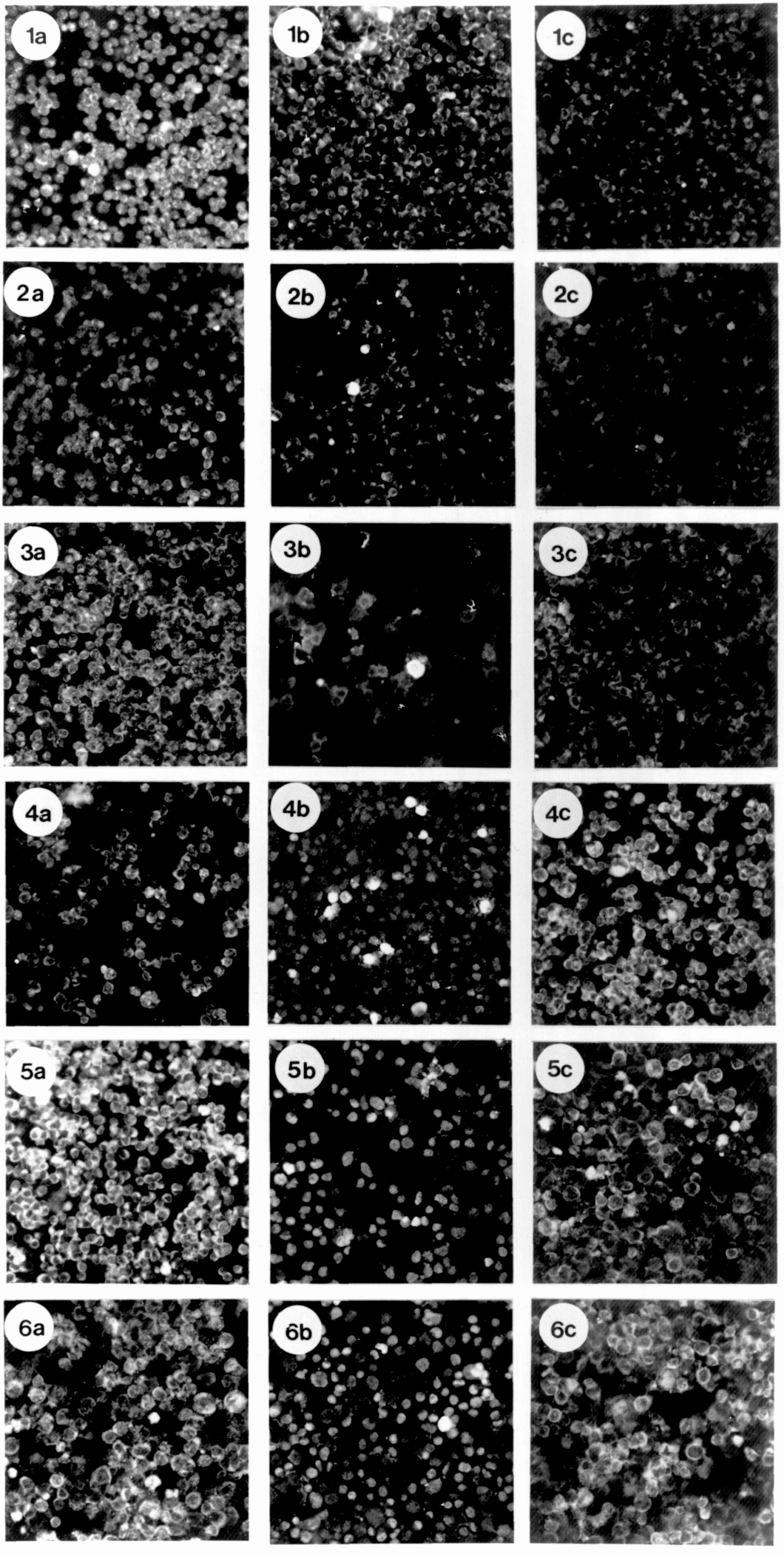
Expression of the EBV lytic cycle antigens, VCA and EA, in IB4 transfectants was determined by indirect immunofluorescence using human antisera JAT (VCA+, EA-), Bull (VCA+, EA+) and WDA (VCA-, EA-). Control cell lines were B95-8, which expresses VCA in 1-5% of cells, and Raji treated with TPA to induce expression of EA in a small proportion of cells. Raji does not express VCA. Photographs were taken on an ultra-violet microscope at x200 magnification. Scale bar represents 100μm. Cell lines are: 1 - B95-8; 2 - Raji treated with TPA; 3 - IB4-HEBo(30/3); 4 - IB4-myc23(24/3); 5 - IB4-myc123(24/3). Antibodies are: a - JAT; b - Bull; c - WDA.


1a
1c
2a
2b
2c
3a
3b
3c
4a
4b
4c
5a
5b
5c

Legend: Figure 31

The effect of TPA on expression of lytic cycle antigens in IB4 transfectants.

IB4-HEBo(30/3) and IB4-myc123(24/3) cells were cultured for 72 hours in growth media containing 20ng/ml TPA, after which time expression of EBV lytic cycle antigens was examined by indirect immunofluorescence using human anti-sera (JAT, VCA+, EA-; Bull, VCA+, EA+; WDA, VCA-, EA-). Parallel cultures of untreated cells were also stained using the same sera. As a control for induction of lytic cycle antigens by TPA, Raji cells were used. Scale bar represents 100μm. Cell lines are: 1 - Raji; 2 - Raji plus TPA; 3 - IB4-HEBo(30/3); 4 - IB4-HEBo(30/3) plus TPA; 5 - IB4-myc123(24/3); 6 - IB4-myc123(24/3) plus TPA. Antibodies are: a - JAT; b - Bull; 3 - WDA


1a
1b
1c
2a
2b
2c
3a
3b
3c
4a
4b
4c
5a
5b
5c
6a
6b
6c

treated cells. In IB4-myc23(24/3) cells no EA positive cells were detected either before or after treatment with TPA.

8.5 Summary

No difference in overall levels of expression of any of the latency associated antigens was observed when cells expressing high levels of c-*myc* were compared to a control cell line. However some differences in distribution of LMP were observed in IB4-myc123(24/3) cells. Vimentin expression was also greatly reduced in these cells and it may be that the unusual distribution of LMP seen in these cells is a consequence of altered vimentin expression.

The lytic cycle antigen, EA, was expressed in a small proportion of parental and pHEBoSV-transfected IB4 cells but not in the *myc*-transfected cells. Treatment with TPA resulted in induction of EA in IB4 and pHEBoSV transfected cells, and in IB4-myc123(24/3), but not in IB4-myc23(24/3). The degree of induction of EA was much greater in the control cells than in the *myc*-transfected cells, indicating that fewer cells were able to enter the lytic cycle in the cell lines expressing high levels of c-*myc*.

9. ASSAYS FOR CELLULAR TRANSFORMATION

9.1 Introduction

Two of the assays classically used for determining cellular transformation are growth in semi-solid medium (Imamura and Moore, 1970), and tumour production in nude mice (Giovanella *et al.*, 1972). BL cell lines are considered tumorigenic in that they generally grow well in soft agar and produce tumours when inoculated subcutaneously into nude mice (Nilsson *et al.*, 1977). In contrast, LCLs do not grow readily in soft agar and do not produce tumours in nude mice (Nilsson *et al.*, 1977). In view of the enhanced growth characteristics of cells constitutively expressing c-*myc* noted earlier in this study, it was of interest to determine whether these characteristics were reflected in a more tumorigenic phenotype.

9.2 Colony formation in soft agar

To assess growth of cell lines in semi-solid medium, 10^4 cells from various cell lines were embedded in 1ml RPMI medium containing 0.8% agar and 20% FCS, and examined daily for the presence of colonies over a 10 day period. A colony

was defined as a clump of 8 or more cells. Each experiment was repeated on at least one other occasion. The BL cell line, Raji, previously reported as growing in soft agar (Nilsson *et al.*, 1977), was used as a positive control. Raji cells grew well in soft agar producing a large number of colonies. In contrast IB4-HEBo(30/3), IB4-myc23(24/3) and IB4-myc123(24/3) cell lines all failed to form colonies (Table 8a). In addition growth in soft agar of IB4-myc23(24/3) clone 46, which, as noted earlier, expresses relatively high levels of c-Myc protein, was assessed. No colonies were observed.

9.3 Tumour production in nude mice

Tumorigenic potential in 4-6 week old female, outbred, athymic, nude mice was assessed by inoculating 5 x 10^6 cells, contained in 0.2ml of PBS, subcutaneously and examining the mice weekly for the presence of tumours (Lombardi *et al.*, 1987). Cells from the IB4-HEBo(30/3) and IB4-myc23(24/3) cell lines were inoculated into athymic nude mice and their ability to form tumours compared to that of Raji cells (Table 8a). Five out of six mice inoculated with Raji cells developed tumours (>1cm in diameter), whereas mice inoculated with IB4-HEBo(30/3) and IB4-myc23(24/3) remained tumour free. In a separate experiment Raji, IB4-HEBo(30/3) and IB4-myc23(24/3) clone 46 cells were inoculated into mice of similar strain, sex and age, using identical experimental conditions. In this experiment (Table 8b) 3 out of 5 mice inoculated with Raji cells developed small tumours (<1cm in diameter), after 3-4 weeks, but these tumours rapidly regressed and by week 7 were no longer visible. Neither the IB4-HEBo(30/3) cell line nor IB4-myc23(24/3) clone 46 developed any signs of tumours. The reasons for the differences in tumorigenic potential of Raji cells in two apparently identical experiments is unclear.

9.4 Summary

From these experiments it appears that, despite their enhanced growth characteristics, EBV-immortalised cells, in which c-*myc* is constitutively expressed, are not necessarily tumorigenic as assessed by the two most commonly used methods.

Table 8

Tumorigenicity of transfected IB4 cell lines

A)

Cell line	Colonies
Raji	+
IB4-HEBo(30/3)	-
IB4-myc23(24/3)	-
IB4-myc123(24/3)	-
IB4-myc23(24/3)Clone 46	-

B)

Experiment I		
Cell line	No. mice innoculated	No. mice with tumours
Raji	6	5
IB4-HEBo(30/3)	5	0
IB4-myc23(24/3)	5	0

Experiment II		
Cell line	No. mice innoculated	No. mice with tumours
Raji	5	3*
IB4-HEBo(30/3)	5	0
IB4-myc23(24/3)Clone 46	5	0

* tumours regressed after 3 weeks

The tumorigenic potential of IB4 transfectants was assessed by growth of cells in soft agar and tumour production in nude mice. A: 104 cells from IB4-HEBo(30/3), IB4-myc23(24/3), IB4-myc123(24/3) and IB4-myc23(24/3) clone 46 cell lines were embedded in growth medium containing 20% FCS and 0.3% agar. After 10 days the cultures were examined for colony formation. As a control, the BL cell line Raji was used. B: Tumorigenic potential in nude mice was assessed by inoculating mice subcutaneously with 5 x 106 cells from the IB4-HEBo(30/3), IB4-myc23(24/3), IB4-myc23(24/3) clone 46 and Raji cell lines. Tumour production was assessed after 8 weeks.

10. EXPERIMENTS WITH OTHER HUMAN LCLS TRANSFECTED WITH pHEBoSV AND pHEBoSVmyc2,3.

10.1 Introduction

To examine whether some of the phenotypic changes observed in IB4 cells constitutively expressing c-*myc* were a general phenomenon of lymphoblastoid cells tranfected with c-*myc*, or were unique to IB4 cells, experiments were carried out on another LCL transfected with pHEBoSV and pHEBoSVmyc2,3. The cell lines examined were CB33HEBoSV (transfected with pHEBoSV) and CB33SVmyc2,3 (transfected with pHEBoSVmyc2,3) (Lombardi *et al.*, 1987). These cell lines were a gift from Prof. A. Rickinson (University of Birmingham). In experiments similar to these reported in this study Lombardi *et al* (1987) had transfected these plasmids into the CB33 LCL, obtained by immortalising cord blood lymphocytes with EBV.

10.2 Expression of c-Myc protein

Levels of c-Myc protein in CB33HEBoSV and CB33SVmyc2,3 were determined by immunoblotting using the Myc1-9E10 mAb, and compared to levels in IB4-HEBo(30/3), IB4-myc123(24/3) and Raji cell lines. As can be seen from figure 32, a marked increase in c-Myc protein was observed in CB33SVmyc23 cells relative to CB33HEBoSV. The level of c-Myc in the *myc*-transfected CB33 LCL was similar to that in IB4-myc123(24/3), and considerably less than that seen in Raji cells.

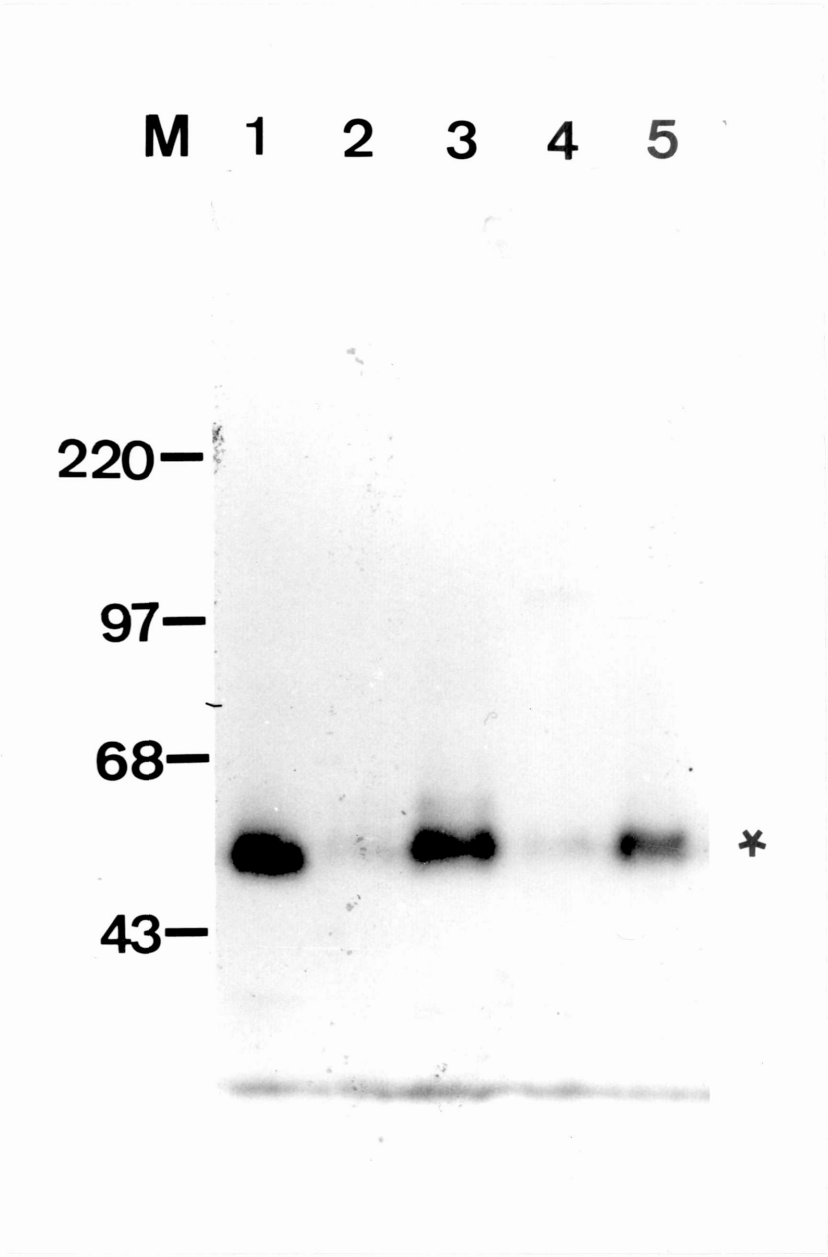
10.3 Expression of cell adhesion molecules

Cell surface expression of CD11a, CD18, CD58 and CD54 on the cell surface was examined using mAbs (MHM24, MHM23, TS2/9 and RR1/1 respectively) and analysed on the PROFILE flow cytometer. Results, from three experiments, demonstrated decreased expression of CD11a (LFA-1 alpha) in the CB33SVmyc2,3 cell line (mean FL/FS = 94.0) relative to CB33HEBoSV (FL/FS = 174.7). Similarly, expression of CD18 (LFA-1 beta) in CB33SVmyc2,3 (FL/FS = 90.4) was considerably lower than that seen in the control cell line, CB33HEBoSV (FL/FS = 153.1) (Table 9, figure 33).

No differences in expression of CD58 (LFA-3) was observed when CB33HEBoSV (FL/FS = 168.6) and CB33SVmyc2,3 (FL/FS = 167.9) were compared. Similarly, expression of CD54 (ICAM-1) was unchanged (CB33HEBoSV, FL/FS = 94.0; CB33SVmyc2,3, FL/FS = 87.1). The LFA-3 and ICAM-1 data are the results of one experiment only (figure 33).

Figure 32

Immunoblot analysis of c-myc protein expression in CB33HEBoSV and CB33SVmyc2,3.



Protein extracted from 2 x 10^6 cells was separated on a 10% polyacrylamide gel in the presence of 0.1% SDS, transferred to nitrocellulose and probed with a c-Myc specific monoclonal antibody (Myc1-9E10). Lane 1, Raji (BL cell line); lane 2, IB4-HEBo(30/3); lane 3, IB4-myc123(24/3); lane 4, CB33HEBoSV; lane 5, CB33SVmyc23; M, high molecular weight protein standards. Position of c-Myc is indicated (*)

Table 9

Flow cytometric analysis of cell adhesion molecule expression in CB33 transfectants

	CB33HEBoSV		CB33myc2,3	
Antigen	Mean FL/FS	S.E.	Mean FL/FS	S.E.
CD11a	174.7	10.3	94.0	6.8
CD18	153.1	6.8	90.4	5.0
CD58	168.6	-	167.9	-
CD54	94.0	-	87.1	-
Negative	13.6	1.3	14.1	3.3

Expression of cell adhesion molecules was assessed by indirect immunofluorescence using monoclonal antibodies. Intensity of staining was analysed on a EPICS PROFILE flow cytometer and expressed as mean linear fluoresence intensity/size ratios (FL/FS) of 5000 cells. As a negative control, cells were stained with second layer FITC conjugated sheep anti-mouse antibody alone. Results are the mean and standard error (S.E.) from three separate experiments, except for CD58 and CD54 which are from one experiment. Antibodies used were: CD11a, MHM24; CD18, MHM23; CD58, TS2/9; CD54, RR1/1.

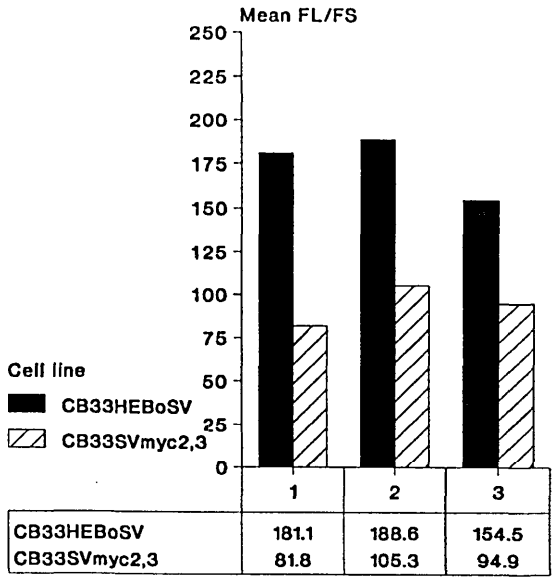
Legend: Figure 33

Flow cytometric analysis of cell adhesion molecule expression in CB33HEBoSV and CB33SVmyc2,3.

Expression of CD11a (LFA-1 alpha subunit), CD18 (LFA-1 beta subunit), CD54 (ICAM-1) and CD58 (LFA-3) in the CB33HEBoSV and CB33SVmyc2,3 cell lines was assessed by indirect immunofluorescence using monoclonal antibodies. The mean fluorescence intensity/size ratio (FL/FS) for 5000 cells was calculated using a PROFILE flow cytometer. CD11a and CD18 histograms represent data from three separate experiments, and the CD54 and CD58 histograms are data from one experiment. Tables below the histograms give the numerical FL/FS values obtained for each experiment. As a negative control, cells were stained with second layer FITC-conjugated anti-mouse IgG alone. A: expression of CD11a. B: expression of CD18. C: negative control experiments for CD11a and CD18. D: expression of CD54, CD58. Antibodies used were: CD11a, MHM24; CD18, MHM23; CD54, RR1/1; CD58, TS2/9.

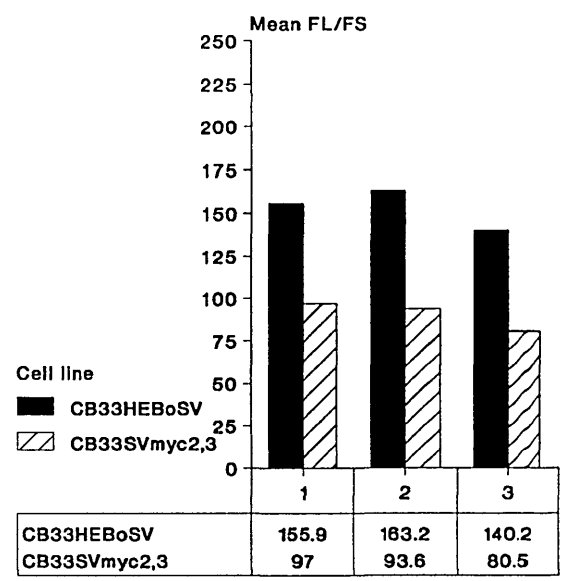
Figure 33

A



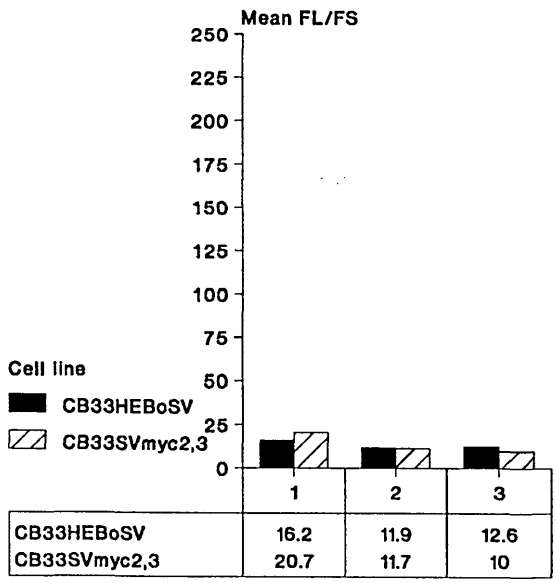
	1	2	3
CB33HEBoSV	181.1	188.6	154.5
CB33SVmyc2,3	81.8	105.3	94.9

B



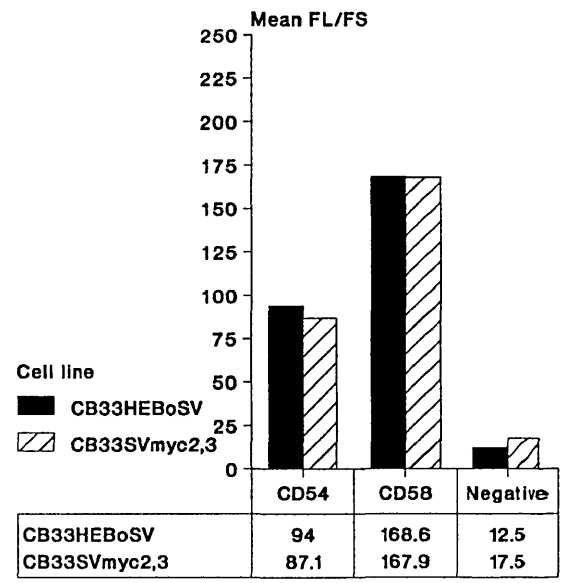
	1	2	3
CB33HEBoSV	155.9	163.2	140.2
CB33SVmyc2,3	97	93.6	80.5

C



	1	2	3
CB33HEBoSV	16.2	11.9	12.6
CB33SVmyc2,3	20.7	11.7	10

D



	CD54	CD58	Negative
CB33HEBoSV	94	168.6	12.5
CB33SVmyc2,3	87.1	167.9	17.5

10.4 Expression of Vimentin and LMP

Expression of LMP and vimentin were examined by immunoblotting and indirect IF, using the CS1-4 pool of mAbs against LMP and the V9 anti-vimentin mAb. No difference in vimentin expression was observed when CB33HEBoSV and CB33SVmyc2,3 were compared by immunoblotting (figure 34). No differences in distribution of either vimentin or LMP was observed by indirect IF. The 'patchy' LMP expression seen in IB4-myc123(24/3) cells was not observed in CB33SVmyc2,3 cells.

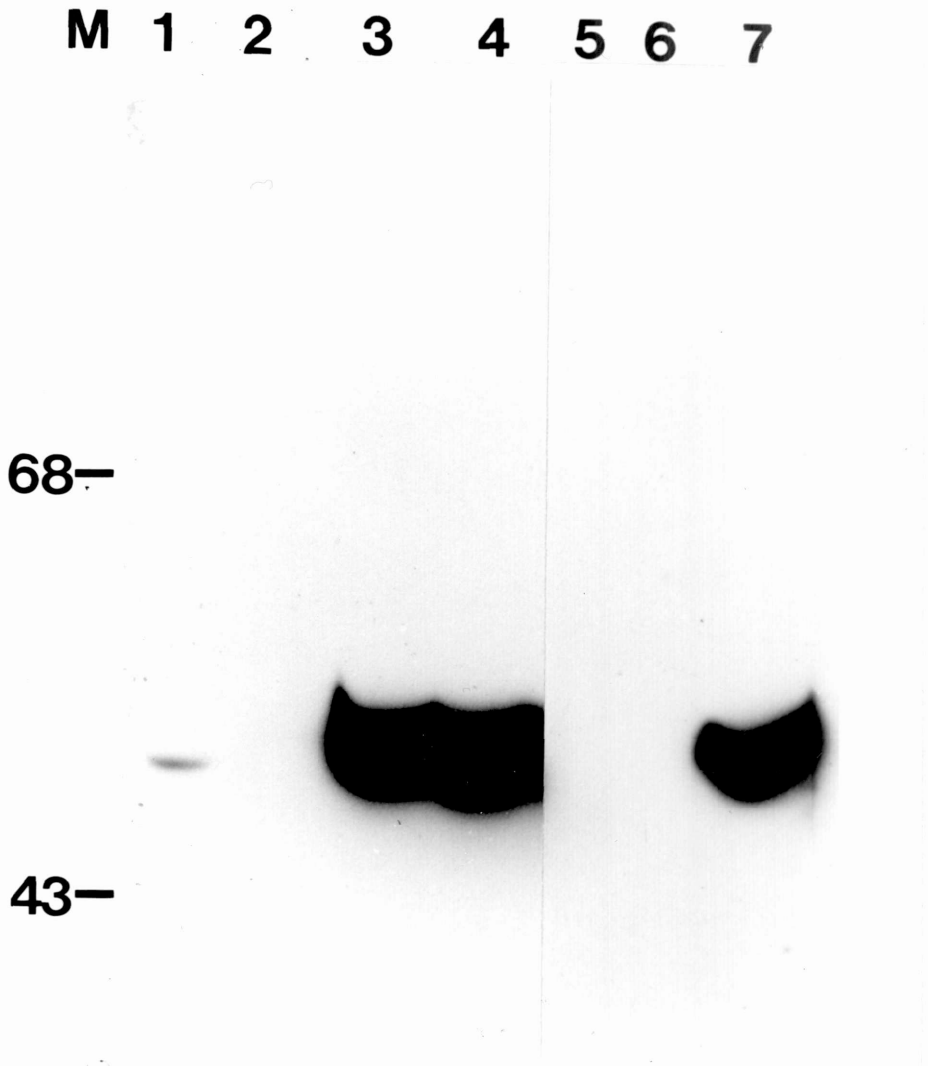
10.5 Summary

An increase in levels of c-Myc protein was observed in the *myc*-transfected CB33 cells, and the amounts of protein detected were comparable to those seen in *myc*-transfected IB4 cells. As with the IB4 transfectants a consistent decrease in expression of LFA-1 was observed in the *myc*-transfected CB33 LCL, relative to the control cell line. Thus, the decreased expression of LFA-1 appears to be a consistent phenomenon in lymphoblastoid cells constitutively expressing c-*myc*.

Unlike the IB4 transfectants, however, no change in vimentin expression was observed, and LMP was uniformly expressed on *myc*-transfected and control cell lines.

Figure 34

Expression of vimentin in CB33HEBoSV and CB33SVmyc2,3.



Protein, extracted from 10^6 cells, was separated on an SDS-PAGE gel (10% acrylamide), transferred to nitrocellulose by western blotting and immunoblotted, using an anti-vimentin mouse monoclonal antibody (V9). Lane 1, Raji (BL cell line); lane 2, ELI (BL cell line); lane 3, CB33HEBoSV; lane 4, CB33SVmyc23; lane 5, Namalwa (BL cell line); lane 6, Ramos (EBV negative BL cell line); lane 7, PS LCL. Positions of high molecular weight standards are indicated (M).

CHAPTER III

RESULTS II

CONSTITUTIVE EXPRESSION OF C-*MYC* IN COMMON MARMOSET LYMPHOBLASTOID CELL LINES: IN VITRO AND IN VIVO STUDIES.

1. INTRODUCTION

The commonly used assays for assessing the tumorigenic potential of cell lines - growth in semi-solid medium and tumour production in nude mice - are somewhat artificial systems for determining the contribution of c-*myc* to the malignant nature of BL cells, in that they bear little resemblence to the environment in which the tumours naturally arise. In addition there are numerous published protocols for nude mice (for example compare, Nilsson *et al.*, 1977; Gurtsevitch *et al.*, 1988) and soft agar experiments (Sugden and Mark, 1977; Nilsson *et al.*, 1977; Lombardi *et al.*, 1987). Sugden and Mark (1977) used a soft agar method, employing a feeder layer of fibroblasts, to isolate proliferating clones of EBV immortalised lymphoblastoid cells. Yet, Nilsson *et al* (1977), using essentially the same method, found that lymphoblastoid cells generally did not proliferate in soft agar, whereas BL cells did. Gursevitch *et al* (1988) also noted inter-experimental variation in tumorigenicity when identical cell lines were inoculated into nude mice.

Several animal models for EBV infection have been examined including owl monkeys (*Aotus trivirgatus*) (Epstein *et al.*, 1973), squirrel monkeys (*Saimiri sciureus*) (Shope and Miller, 1973), cotton-top tamarins (*Saguinus oedipus oedipus*) (Shope *et al.*, 1973), gibbons (*Hylobates lar*) (Werner *et al.*, 1972) and common marmosets (*Callithrix jacchus*) (Wedderburn *et al.*, 1984). The animal model which has been most commonly used for studying the biology of EBV is the cotton-top tamarin, and it is this species which has played a central role in work to develop a vaccine against EBV (Epstein and Morgan, 1986). Inoculation of these animals with EBV results in the rapid appearance of lymphomas (Shope *et al.*, 1973a; Epstein *et al.*, 1985). Appearance of these lymphomas, which phenotypically resemble EBV-associated post transplant lymphomas rather than BL (Cleary, *et al.*, 1985; Young *et al.*, 1989b; Allday *et al.*, 1990), can be prevented by vaccination with either purified cell membranes of EBV infected cells or with a purified subunit of the EBV membrane antigen (gp340) (Epstein *et al.*, 1985). Although undoubtedly of value in development of vaccines, the cotton-top tamarin's response to infection with EBV is unlike humans, in whom EBV-associated malignant disease is a very rare event. As a better model for the studying the role of c-*myc* in EBV associated human

malignant disease the common marmoset was considered. When inoculated with EB virus preparations, or LCLs, common marmosets appear to undergo a primary asymptomatic EBV infection, as demonstrated by the appearance of IgG class antibodies to VCA which remain detectable for the lifespan of the animal (Dr N. Wedderburn, Royal College of Surgeons, personal communication). Heterophile antibodies and IgG antibodies against EA have also been detected in some common marmosets infected with EBV (Wedderburn *et al.*, 1984). Both of these markers are typically found in infectious mononucleosis in humans (chapter I, section 5.7.1). Unlike cotton-top marmosets, common marmosets do not develop any lymphoproliferative or malignant lesions (Miller, 1979; Wedderburn *et al.*, 1984). Whilst the life-long persistence of anti-VCA IgG antibodies is suggestive of a persistent infection, spontaneous EBV immortalised cell lines have not been established from the peripheral blood of infected marmosets, nor has virus been recovered from throat washings (Miller, 1979; Wedderburn *et al.*, 1984). Thus, at least in respect to the serological response, infection of common marmosets mimics the asymptomatic infection seen in humans (Niederman *et al.*, 1970; Henle W., and Henle G., 1979), and represents a better model than cotton-top marmosets for studying *in vivo* the role of c-*myc* in the pathogenesis of BL.

2. EXPERIMENTAL DESIGN

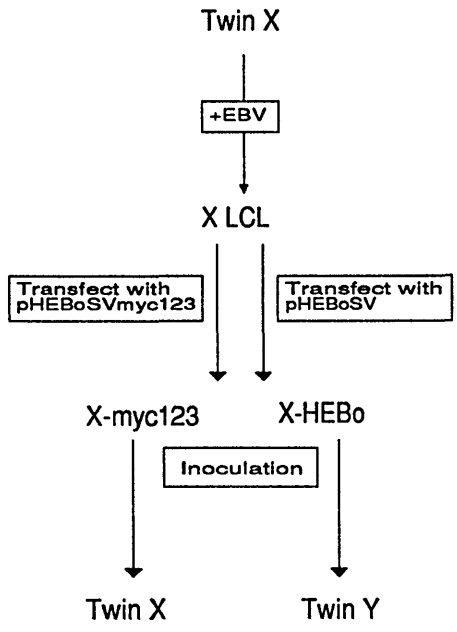
The plan of investigation used in this set of experiments is outlined in figure 35. The original plan was to take peripheral blood from one of a set of marmoset twins, infect with EBV to generate a LCL and transfect cells with pHEBoSV and pHEBoSVmyc123. Having established and characterised stable transfected cell lines, the *myc*-transfected cell line would be inoculated into the host animal, and the control cell line into the other twin. Marmosets from multiple births are haematologically chimeric as a result of anastomoses between placentas (Hetherington *et al.*, 1981). They are thus tolerised to each others haemopoietic cells, and no host versus graft problems should occur. The animals would be monitored at regular intervals for signs of malignant disease, and samples taken for EBV-specific serological, and haematological, studies.

One set of marmoset triplets (Marmosets number 244, 245 and 246) and one set of twins (Marmosets number 286 and 287) were obtained and housed at the National Institute for Biological Standards and Controls, under the supervision of Dr N. Wedderburn (Royal College of Surgeons, London).

Figure 35

In vivo tumorigenic potential of _myc_-transfected marmoset lymphoblastoid cells: experimental design

Diagram showing design of proposed experiment involving common marmosets as a model for the *in vivo* tumorigenic potential of *myc*-transfected cells. An EBV immortalised lymphoblastoid cell line (LCL) from marmoset twin X (X LCL) would be transfected with i) pHEBoSV to create the X-HEBo control cell line and, ii) with pHEBoSVmyc1,2,3 to create X-myc123. X-myc123 would be inoculated into twin X and X-HEBo into twin Y, and the marmosets monitored for the appearance of tumours.



3. TRANSFECTION OF COMMON MARMOSET LCLS

3.1 Sensitivity of Common Marmoset LCLs to hygromycin B

LCLs from marmosets 245 and 287 (M245 and M287) were obtained from Dr N Wedderburn. These lines were established by infection with virus from the M81 common marmoset lymphoblastoid cell line (Desgranges *et al.*, 1976). Sensitivity to hygromycin B was assessed exactly as described previously (chapter II, section 2.3). Both M245 and M287 LCLs proved highly sensitive to hygromycin B with only 50μg/ml being required to inhibit proliferation, as assessed by incorporation of tritiated thymidine. These results were confirmed by trypan blue staining of cells cultured for 10 days in medium containing 50μg/ml hygromycin B.

3.2 Transient expression of SV40 large T antigen

To determine voltage and capacitance settings for optimal electroporation of the marmoset LCLs, transient expression assays were performed using the pSV3Neo plasmid and staining for the presence of SV40 LT, by indirect IF, after 48 hours. Results of transient electroporation assays on M245 and M287 LCLs, in addition to those from 2 other common marmoset LCLs M232 and M242 are presented in Table 10. Expression of SV40 LT was detected in pSV3Neo transfected M232 cells in a small number of cells (<0.1%) using a number of different voltage and capacitance combinations, indicating that the SV40 early region promoter/enhancer functions in common marmoset cells. However, despite using a variety of voltage and capacitance settings, no transient expression of SV40 LT was observed in M242, M245 or M287 cell lines. So, whilst it was possible to transfect one common marmoset LCL by electroporation, the cell lines of interest in these experiments - M245 and M287 - were not susceptible to electroporation.

Following the lack of success with electroporation, liposome-mediated transfection was attempted, using a commercially available reagent, Lipofectin (Gibco). The method used was essentially as described by the manufacturers, and is described in detail in the Materials and Methods section. Briefly, cells were attached to the surface of plastic petri dishes and incubated with serum free medium containing 10μg pSV3Neo and 10μg Lipofectin for 5 hours. Cells were removed from the plastic using trypsin and cultured in normal growth medium for 48 hours before being stained for the presence of

Table 10

Transient expression of SV40 large T antigen in pSV3Neo transfected M232, M242, M245 and M287 LCLs

10^6 cells from four different common marmoset LCLs (M232, M242, M245 and M287) were electroporated with 10μg pSV3Neo DNA using a variety of different voltage (kV) and capacitance (μFd) combinations. Electroporated cells were cultured for 48 hours and stained, by indirect immunofluorescence, for expression of the pSV3Neo encoded SV40 large T antigen (LT). Symbols represent; <0.1% cells expressing LT (+), no cells expressing LT (-), no viable cells after 48 hours in culture (*).

M245 LCL			M287 LCL		
KV	μFD	LT	KV	μFD	LT
0.25	960	-	0.33	960	-
0.26	960	-	0.35	250	-
0.27	960	-	0.35	500	-
0.29	960	-	0.35	960	-
0.30	125	-	0.36	250	-
0.30	250	-	0.36	500	-
0.30	500	-	0.36	960	-
0.31	960	-	0.39	250	-
0.33	960	-	0.39	500	-
0.35	125	-	0.39	960	-
0.35	250	-	0.40	250	-*
0.35	500	-*	0.40	500	-*
0.35	960	-*	0.40	960	-*
0.37	960	-*	0.45	250	-*
0.39	960	-*	0.45	500	-*
0.40	125	-	0.45	960	-*
0.40	250	-*	1.00	25	-
0.40	500	-*	1.20	25	-
0.41	960	-	1.40	25	-
0.43	960	-*	1.60	25	-
0.45	250	-*	1.80	25	-
0.45	960	-*	2.00	25	-
			2.50	0.25	-

Table 10 contd.

M232 LCL			M242 LCL		
KV	µFD	LT	KV	µFD	LT
0.10	960	-	0.25	0.25	-
0.20	960	-	0.25	1.0	-
0.30	960	-	0.25	25	-
0.25	500	+	0.25	500	-*
0.45	125	-	0.45	0.25	-
0.45	250	-	0.45	1.0	-
0.45	500	-	0.45	25	-
0.45	960	-	0.45	500	-*
0.50	0.25	-	0.50	0.25	-
0.50	1.0	-	0.50	1.0	-
0.50	25	+	0.50	25	-
0.50	500	+	0.50	500	-
0.75	0.25	-	0.75	0.25	-
0.75	1.0	-	0.75	1.0	-
0.75	25	+	0.75	25	-
0.75	500	-	0.75	500	-
1.0	0.25	-	1.0	0.25	-
1.0	1.0	-	1.0	1.0	-
1.0	3.0	-	1.0	25	-
1.0	25	-	1.0	500	-
1.0	500	-			
0.25	1.0	-			
0.25	25	-			

SV40 LT. SV40 LT was detected in a small proportion of M245 cells (<0.1%) but not in any of the M287 cells, so subsequent experiments concentrated on using Lipofectin to introduce pHEBoSV and pHEBoSVmyc1,2,3 into M245 cells.

3.3 Establishment of stable transfected cell lines by liposome-mediated transfection

Transfection of M245 cells with pHEBoSVmyc1,2,3, using Lipofectin, resulted in the establishment of one hygromycin B resistant cell line designated M245-myc123, which was expanded and maintained in culture with growth medium containing hygromycin B (50μg/ml). Attempts to transfect M245 cells with pHEBoSV using the same method proved unsuccessful. In the absence of a pHEBoSV-transfected M245 cell line untransfected M245 cells were used as a control.

4. EXPRESSION OF THE TRANSFECTED MYC GENE

4.1 Transcription of the exogenous c-*myc* gene

To confirm that the c-*myc* gene was being expressed from the transfected pHEBoSVmyc1,2,3 plasmid the polymerase chain reaction (PCR) was used to detect plasmid-derived transcripts and differentiate them from endogenous transcripts. cDNAs were made by reverse transcription from total cellular RNA isolated from M245 and M245-myc123 LCLs. These cDNAs were then used in the PCR reaction. Oligonucleotide primers (20mers) situated in the SV40 early region promoter and exon II of c-*myc* were used (figure 36). This allowed differentiation of amplified product from the cDNAs (674bp) and any contaminating plasmid DNA (2298bp). Reaction conditions are given in chapter VI, section 10.3.

As can be clearly seen amplified sequences, of the correct size (674bp), are present in the cDNA generated from M245-myc123 RNA but not in the control M245 cell line (figure 37a). These sequences hybridised to ^{32}P labelled pHEBoSV-myc1,2,3 DNA (figure 37b) demonstrating conclusively that the plasmid derived c-*myc* gene is being transcribed in the transfected cells.

Legend: Figure 36.

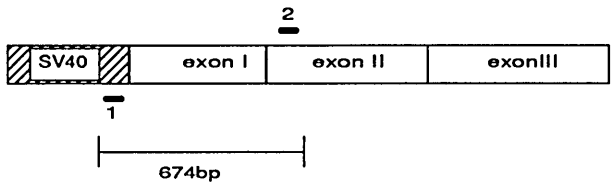
Oligonucleotide primers used in polymerase chain reaction.

Diagram showing location of primers used in polymerase chain reaction (PCR) detection of c-*myc* sequences derived from pHEBoSV-myc1,2,3. Use of primers in the SV40 early region enhancer/promoter element and c-*myc* exon II allowed discrimination of DNA amplified from cDNA obtained by reverse transcription of RNA (A) and contaminating plasmid DNA (B). The sequences of the oligonucleotide primers are given in C.

Figure 36

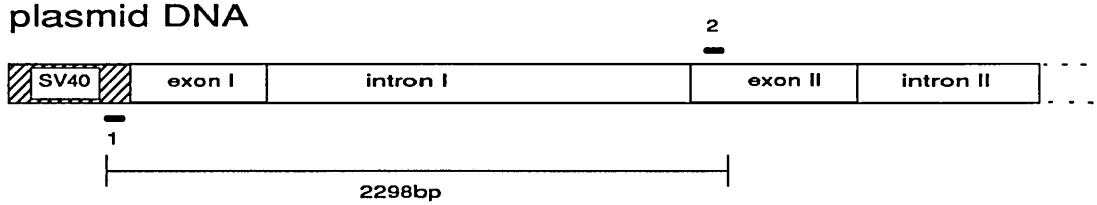
A

cDNA



B

plasmid DNA



C

Primer 1: GCTATTCCAGAAGTAGTGAG [nucleotides 15-34 in Fiers et al, 1978]

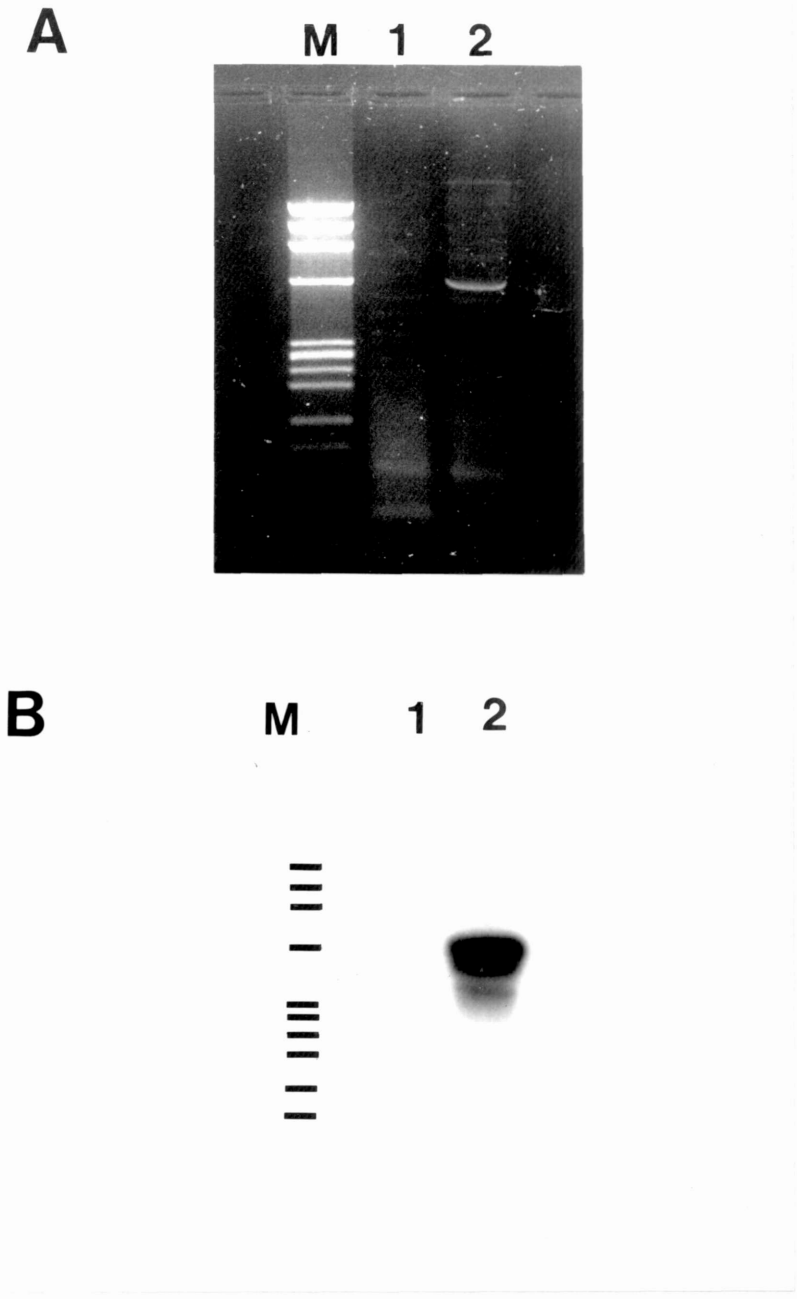
Primer 2: CGAGGTCATAGTTCCTGTTG [nucleotides 4544-4563 in Gazin et al, 1984]

Legend: Figure 37

Detection of plasmid-derived c-*myc* transcripts in M245-myc123 using polymerase chain reaction (PCR)

cDNAs were generated by reverse transcription from total cellular RNA isolated from M245 and M245-myc123. Primers located in the SV40 early region promoter and exon II of c-*myc* were annealed to the cDNAs and sequences between the two primers amplified by PCR. The PCR products were electrophoresed on an agarose gel, transferred to a nylon membrane and probed with a ^{32}P labelled double stranded DNA probe for c-*myc*. A: ethidium bromide stained gel prior to transfer to nylon membrane. B: nylon membrane probed for c-*myc* sequences. Track 1, M245; track 2, M245-myc123. Positions of molecular weight markers generated by digestion of phiX174 DNA with the restriction endonuclease HaeIII are indicated (M). Sizes of markers, in kb from the top of the gel are; 1.35, 1.08, 0.87, 0.60, 0.31, 0.28/0.27, 0.23, 0.19.

Figure 37



4.2 C-Myc protein expression

Levels of c-Myc protein in M245 and M245-myc123 cell lines were compared by immunoblotting. Protein extracts from 2 x 10^6 cells for each cell line were separated on an SDS-PAGE gel, transferred to nitrocellulose and probed with the Myc1-9E10 mAb against c-*myc*. Protein from equivalent numbers of Raji, IB4-HEBo(30/3), IB4-myc23(24/3) and IB4-myc123(24/3) was also immunoblotted for comparison.

The results of immunoblotting with the anti-c-*myc* antibody, which were repeatable on a number of separate occasions, are presented in figure 38. A considerable increase in protein expression in the M245-myc123 cell line relative to M245 was observed. The difference in expression, and the levels of, c-Myc protein of M245 and M245-myc123 are comparable to that seen in control and *myc*-transfected IB4 cells. As with IB4 and CB33 transfectants, the levels of protein are considerably less than that in the BL cell line, Raji.

5. MORPHOLOGY AND GROWTH CHARACTERISTICS

5.1 Morphology and growth pattern

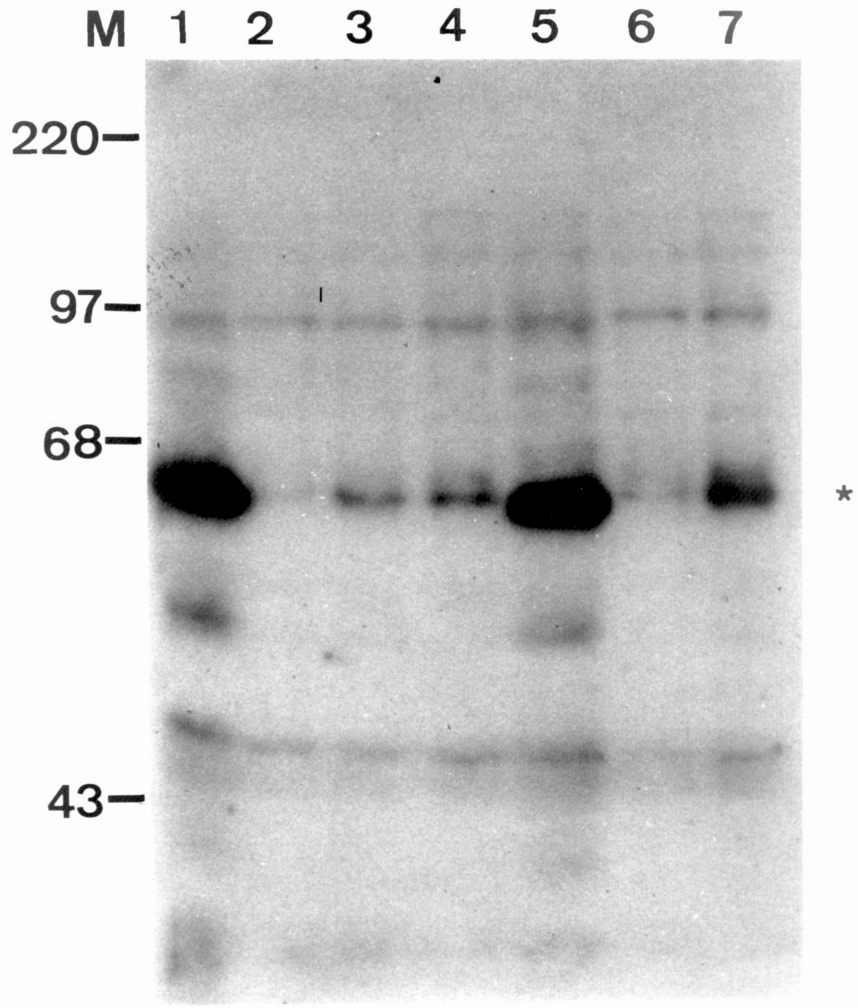
As with the IB4 transfectants, no morphological differences between M245-myc123 and M245 were observed under a light microscope. Unlike the IB4 transfectants, no difference in growth pattern was observed, with both cell lines demonstrating homotypic cell adhesion, growing in large clumps of cells.

5.2 Growth kinetics

The growth of *myc*-transfected and control M245 cells in normal growth medium (containing 10% FCS) was assessed by seeding cells at a density of 2 x 10^5/ml in growth medium and leaving the cells for 14 days, with no replacement of medium. Cell proliferation was monitored by counting triplicate aliquots of cells in the presence of trypan blue at regular intervals. The results, from a single experiment, are presented in figure 39. The doubling time of *myc*-transfected and non-transfected cells was similar with approximately 2 days required for cells to double in number. Cells from the control LCL reached a maximum cell density of 6.5 x 10^5/ml by day 7. M245-myc123 cells reached a higher cell density of 11.2 x 10^5/ml on day 4.

Figure 38

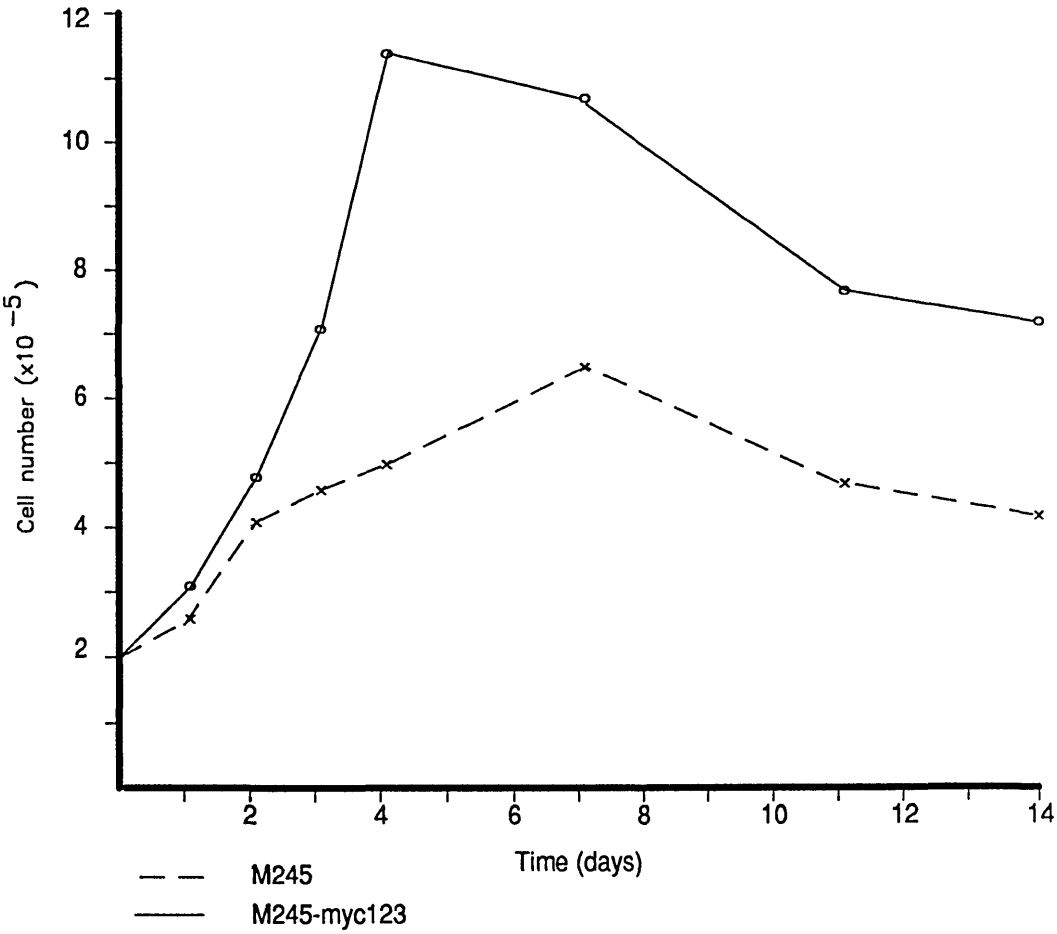
Immunoblot analysis of c-Myc protein expression in *myc*-transfected and untransfected common marmoset cells.



Protein extracts from 2 x 10^6 cells were separated on an SDS-PAGE gel, transferred to nitrocellulose and probed with an anti-c-Myc monoclonal antibody (Myc1-9E10). Protein from M245-myc123 (track 7) was compared to M245 (track 6) and Raji (tracks 1 and 5). For comparison protein from equivalent numbers of IB4-HEBo(30/3), IB4-myc23(24/3) and IB4-myc123(24/3) (tracks 2, 3 and 4 respectively) was probed with the same antibody. Positions of c-Myc (*) and pre-stained molecular weight standards (M) are indicated.

Figure 39

Growth of M245 and M245-myc123 LCLs in normal growth medium.



Growth of M245 and M245-myc123 LCLs was assessed by resuspending cells in normal growth medium at a density of 2 x 10^5/ml and culturing for 14 days, with no addition of fresh medium. Cell proliferation was assessed by counting cells in the presence of trypan blue at regular intervals. Data is from one experiment.

5.3 Serum dependency

Growth of M245 and M245-myc123 in reduced serum concentrations was assessed by culturing cells in growth medium containing 1% FCS and assessing incorporation of tritiated thymidine after 3 days, as described for IB4 cell lines (chapter II, section 4.5). Data from 3 separate experiments are presented in figure 40. As with the IB4 transfectants, a considerable difference in levels of thymidine incorporation was observed when M245 (mean cpm = 17641) and M245-myc123 (mean cpm = 69182) cell lines were compared.

5.4 Summary

As in human lymphoblastoid cells, high level expression of c-*myc* results in a reduced growth factor requirement, which could provide the cells with a growth advantage over normally regulated cells. The growth kinetics data are from one experiment only but results similar to those seen in *myc*-transfected IB4 cells were observed, with M245-myc123 cells growing to a higher cell density in normal growth medium, compared to control cells.

No differences in cell morphology or growth pattern were observed. Little is known about expression of cell adhesion molecules and the mechanisms of homotypic B-cell adhesion in common marmosets, but no reactivitity was observed when M245 cells were stained with a mAb (MHM24) against CD11a. This suggests that either the mAb against the human LFA-1 molecule does not recognise the marmoset LFA-1 molecule, or alternatively, that LFA-1 is not expressed on marmoset cells.

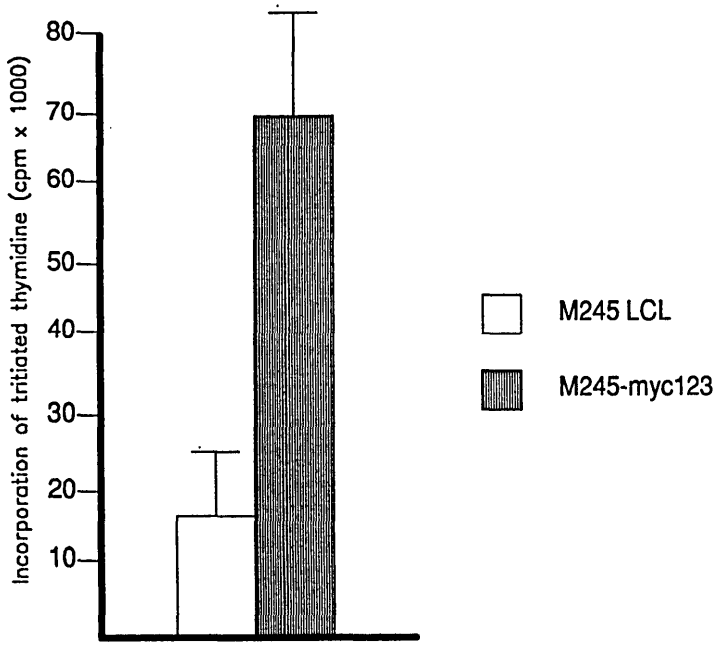
6. GROWTH OF CELLS IN SOFT AGAR AND TUMOUR PRODUCTION IN NUDE MICE

6.1 Colony formation in soft agar

To assess growth of the *myc*-transfected and control cell lines in semi-solid medium 10^4 cells were embedded in 1ml growth medium containing 0.8% agar and 20% FCS. The Raji cell line was used as a control. Neither M245-myc123 or M245 cell lines grew in colonies in soft agar, in contrast to the Raji cell line, which produced large numbers of colonies (Table 11).

Figure 40

Uptake of tritiated thymidine by M245 and M245-myc123 cell lines cultured in low serum concentrations



Proliferation of M245 and M245-myc123 in low serum concentrations was assessed by culturing cells for two days in 1% foetal calf serum and measuring incorporation of tritiated thymidine during a four hour period. Results are the mean and standard error of three separate experiments.

Table 11

Tumorigenicity of M245 LCL and M245-myc123

A) Growth in soft agar

Cell line	Colonies
Raji	+
M245	-
M245-myc123	-

B) Tumour production in nude mice

Cell line	No. mice innoculated	No. mice with tumours
Raji	5	3*
M245 LCL	5	0
M245-myc123	5	0

* tumours regressed after 3 weeks

The tumorigenic potential of M245 and M245-myc123 was assessed by growth of cells in soft agar and tumour production in nude mice. A: 10^4 cells from M245 and M245-myc123 LCL were embedded in growth medium containing 20% FCS and 0.8% agar. After 14 days the cultures were examined for colony formation. As a control, the BL cell line Raji was used. B: Tumorigenic potential in nude mice was assessed by inoculating mice subcutaneously with 5 x 10^6 cells from M245, M245-myc123 and Raji cell lines. Mice were monitored for 8 weeks.

6.2 Tumour production in nude mice

Four to six week old female athymic nude mice were inoculated subcutaneously with 5 x 10^6 M245 LCL, M245-myc123 and Raji cells. Five mice were inoculated for each cell line, and the animals were examined weekly for the presence of lesions. As noted in the experiments with IB4 transfectants (chapter II, section 9.3), 3 out of 5 nude mice inoculated with Raji cells developed small tumours (<1cm^3) after 4 weeks. However, by week 7, these tumours were no longer visible. No tumours were observed in mice inoculated with either the M245 or the M245-myc123 cell line.

6.3 Summary

In results similar to those observed earlier with *myc*-transfected IB4 cells, no evidence for a tumorigenic phenotype in marmoset lymphoblastoid cells, expressing high levels of c-*myc* was observed. The data obtained from the nude mice experiments should be interpreted with caution as only transient tumours were seen in the control animals inoculated with Raji cells.

7. INOCULATION OF COMMON MARMOSET CELL LINES INTO SYNGENEIC ANIMALS

7.1 Inoculation of cell lines

Marmosets 245 and 246 were inoculated intra-venously with 5 x 10^7 M245-myc123 cells. An equal number of cells were inoculated intra-peritoneally at the same time. As a control, marmoset number 244 was inoculated via the same routes using an equal number of M245 cells (figure 41). All invasive work on the animals was carried out by Dr N. Wedderburn (Royal College of Surgeons, London).

7.2 EBV serology

Antibody titres to EBV antigens were determined by indirect IF; using acetone fixed P3HR-1 cells to titrate anti-VCA antibodies, and acetone-fixed TPA treated Raji cells to titrate anti-EA antibodies. Results of EBV specific serology on the 3 marmosets are summarised in Table 12. All 3 marmosets were EBV seronegative prior to inoculation with cell lines, indicating that they had not previously been infected with EBV. All developed anti-VCA IgM responses within 7 days of inoculation, and anti-VCA IgG class antibodies to VCA were

Figure 41

Inoculation of common marmosets with M245 and M245-myc123 LCL: experimental procedure

Diagram to show the procedure of the experiment to determine the tumorigenic potential of common marmoset lymphoblastoid cells transfected with pHEBoSVmyc1,2,3. An EBV immortalised LCL generated from the peripheral blood of marmoset 245 (M245 LCL) was transfected with pHEBoSVmyc1,2,3 and a stable hygromycin B resistant cell line (M245-myc123) established. 10^8 cells from this line were inoculated intravenously (i.v.) and intra-peritoneally (i.p.) (5 x 10^7 cells via each route) into marmosets 245 and 246. As a control, 10^8 cells from the M245 LCL were inoculated, via the same routes, into marmoset 244. Peripheral blood samples were taken at regular intervals from each animal for EBV serology and for leucocyte total and differential cell counts. The marmosets were also examined frequently for signs of malignant disease.

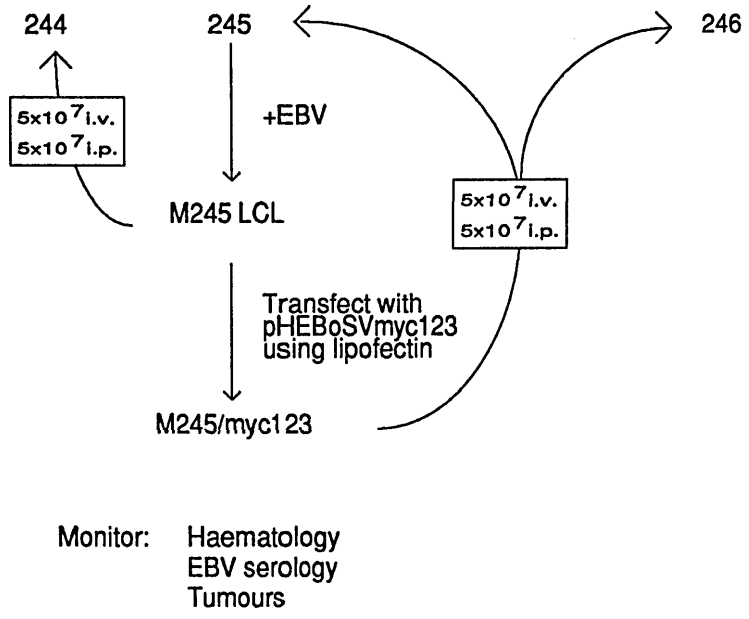


Table 12

EBV-specific serology in marmosets inoculated with M245 LCL and M245-myc123 LCL.

A) Marmoset 244

Days p.i.	VCA IgG	VCA IgA	VCA IgM	EA IgG
-34	<8	NT	<8	<8
7	<8	<8	128	<8
14	8	<8	64	<8
21	8	<8	8	<8
28	16	<8	<8	<8
42	32	<8	<8	<8
168	16	NT	NT	<8

B) Marmoset 245

Days p.i.	VCA IgG	VCA IgA	VCA IgM	EA IgG
-34	<8	NT	<8	<8
7	<8	<8	128	<8
14	<8	<8	32	<8
21	8	<8	16	<8
28	8	<8	<8	<8
42	16	<8	<8	<8
168	32	NT	NT	<8

C) Marmoset 246

Days p.i.	VCA IgG	VCA IgA	VCA IgM	EA IgG
0	<8	NT	NT	<8
7	<8	<8	8	<8
14	8	<8	16	<8
21	16	<8	<8	<8
35	32	<8	<8	<8

Tables showing reciprocal antibody titres to EBV viral capsid antigen (VCA) and early antigen (EA) complexes. Serum samples were taken from the marmosets at regular intervals following inoculation of the cell lines, and antibody titres to VCA and EA were assessed by indirect immunofluorescence on P3HR-1 fixed cells (for antibodies to VCA) and TPA-treated Raji cells (for antibodies to EA). A: marmoset 244 (inoculated with M245 LCL). B: marmoset 245 (inoculated with M245-myc123). C: marmoset 246 (inoculated with M245-myc123). NT - not tested; p.i. - post inoculation; < - less than.

detectable 14-21 days after inoculation. IgM class antibodies to VCA fell below detectable levels after 28 days, and anti-VCA IgG remained detectable in all animals throughout the experiment. IgG class antibodies to EA were not detectable at any stage. Similarly, anti-VCA IgA antibodies were not detected.

7.3 Haematology

The haematological status of the 3 marmosets was examined in two ways. Firstly, a total white cell count (WCC) was performed at regular intervals to look for any evidence of lymphocytosis. Secondly, differential leukocyte counts were performed by making thin films of peripheral blood on glass slides, fixing in methanol and staining with Giemsa stain. The differential leucocyte counts were carried out by Dr. I. Reilly (Dept of Haematology, Hammersmith Hospital, London).

The haematological data from the 3 marmosets are summarised in Table 13. No significant increase in total white cell count was observed in the control marmoset (244) over a period of 24 weeks following inoculation. On morphological grounds the haematological picture of marmoset 244 remained relatively stable with the only unusual feature being a transient appearence of atypical, lymphoblastoid-like cells 70 days post-inoculation which, at their peak, represented 15% of the total WCC. However, these did not persist in the circulation, and had virtually dissappeared by day 84. In contrast, marmoset 245 - inoculated with M245-myc123 - developed a large number of atypical, lymphoblastoid-like, mononuclear cells soon after inoculation. These reached as high as 49% of the total white cell count on day 63, and were still as high as 13% on day 168. Figure 42 shows the appearance of these atypical lymphocytes. Despite this abnormality in marmoset 245, the total white cell count was not dramatically raised, although a 2-fold increase was observed soon after inoculation and the count did not return to pre-inoculation levels until day 84. In the other marmoset inoculated with the *myc*-transfected cell line (246), the haematological picture was similar to that seen in the control animal, with only low numbers of atypical lymphocytes detected.

7.4 Tumorigenic potential of cell lines

The inoculated marmosets were examined weekly for indications of malignant lesions. The animals were weighed, and the spleen and lymph nodes examined

Table 13

Haematological data on common marmosets inoculated with M245 LCL and M245-myc123 LCL

Peripheral blood from the set of marmoset triplets was taken at regular intervals following inoculation with cells from the M245 LCL or M245-myc123 LCL and total white cell counts (WCC) performed. Thin blood films were also prepared, stained with Giemsa, and differential white cell counts performed. A: marmoset 244 (inoculated with M245 LCL). B: marmoset 245 (inoculated with M245-myc123). C: marmoset 246 (inoculated with M245-myc123). p.i. - post inoculation; WCC - white cell count; PMN - polymorphonuclear cells; MN - mononuclear cells; Mono. - monocytes; Lymph. - lymphocytes; ND - not done.

A) Marmoset 244

Days p.i.	Total WCC $x10^6$/ml	PMN(%)	Lymph.(%)	Mono.(%)	Atypical MN (%)
-34	4.3	69	31	0	0
0	ND	65	31	4	0
7	3.2	74	23	3	0
14	4.0	60	31	9	0
21	3.4	69	30	1	0
28	2.0	50	46	4	0
42	3.0	57	41	0	2
56	3.2	54	42	3	1
63	ND	70	27	3	0
70	2.0	64	18	1	15
84	2.9	65	29	2	3
111	3.1	78	15	1	2
137	ND	81	17	2	0
158	ND	72	22	6	0
179	ND	54	36	8	2

Table 13 contd.

B) Marmoset 245

Days p.i.	Total WCC x10^6/ml	PMN(%)	Lymph.(%)	Mono.(%)	Atypical MN (%)
-34	4.0	46	52	2	0
0	ND	52	44	4	0
7	8.4	26	61	6	7
14	6.2	38	41	15	6
21	6.6	29	56	1	14
28	5.2	39	50	1	9
42	9.2	39	52	0	10
56	6.0	41	38	1	20
63	ND	32	18	1	49
70	7.2	34	22	1	43
84	5.2	32	41	2	25
111	4.9	28	49	0	23
137	ND	54	29	5	12
158	ND	30	47	10	13
179	ND	40	43	4	13

C) Marmoset 246

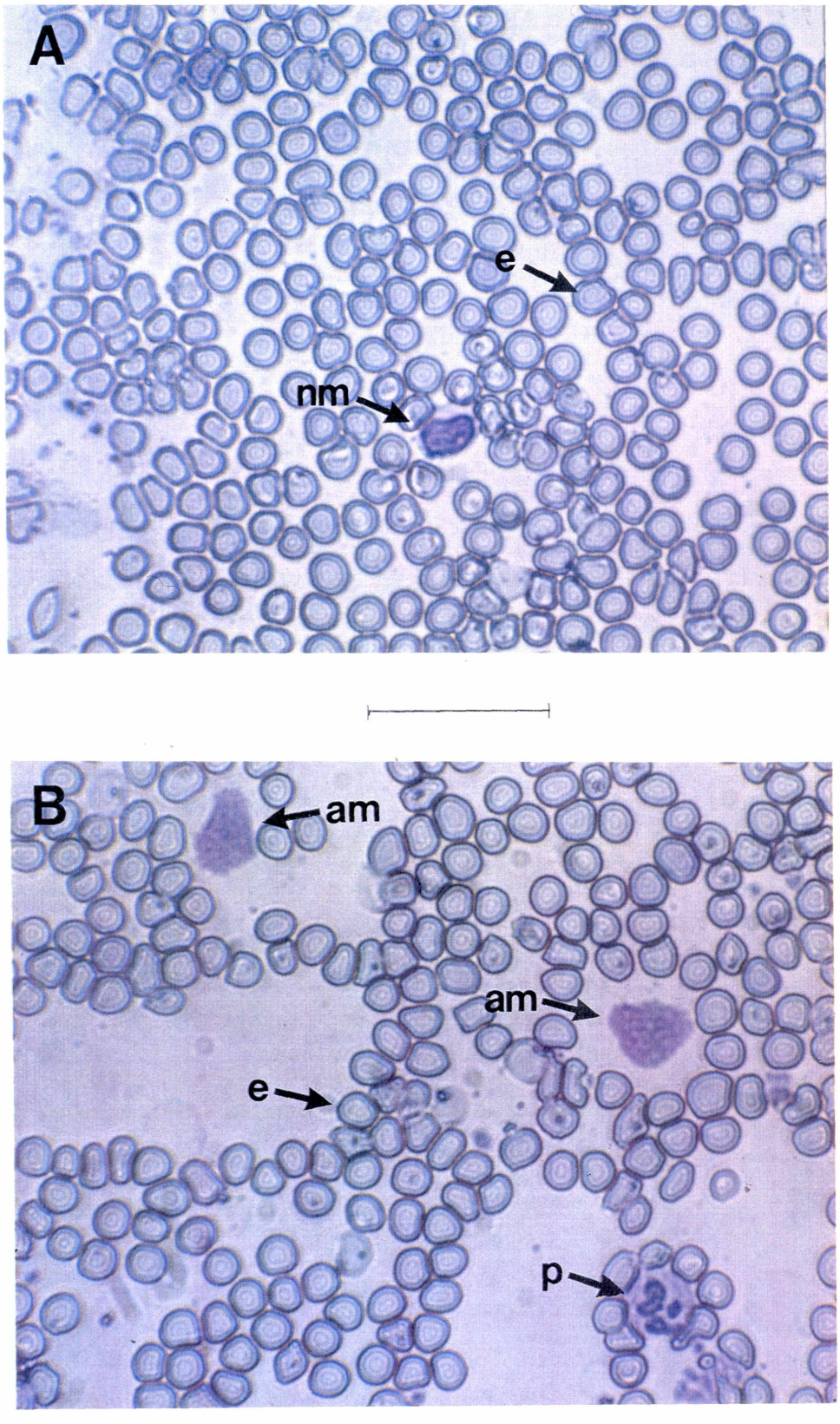
Days p.i.	Total WCC x10^6/ml	PMN(%)	Lymph.(%)	Mono.(%)	Atypical MN (%)
-146	5.0	69	31	0	0
0	4.9	46	43	2	9
7	6.6	73	22	1	4
14	3.1	54	41	0	5
21	3.8	55	42	1	3
35	6.0	53	39	3	5
81	ND	67	30	0	3
105	ND	52	43	3	2
126	ND	55	44	0	1

Figure 42

Morphological appearance of mononuclear cells in peripheral blood of common marmosets.

Photomicrographs showing morphology of mononuclear cells from the peripheral blood of, A: marmoset 244 (inoculated with M245 LCL) and, B: marmoset 245 (inoculated with M245-myc123 LCL). Methanol fixed thin blood films were stained with Giemsa and examined under a light microscope. am - atypical mononuclear cells; nm - normal mononuclear cells; p - polymorphonuclear cells; e - erythrocytes. Scale bar = 40μm

Figure 42



for abnormalities. Over the 6 month period of this experiment no signs of malignant disease was observed. All marmosets were healthy, with no dramatic changes in weight noted. This does not rule out the possibility of an early, or pre-malignant, disease being present but undetected.

7.5 Summary

These data demonstrate a seroconversion to EBV in all 3 marmoset following inoculation of 10^8 EBV infected cells. If the seroconversion is a consequence of infection with EBV, then the source of virus was presumably virus released from the proportion of the introduced cells in the lytic cycle. Indirect IF of M245 and M245-myc123 cells revealed approximately 1-5% of cells expressing VCA, and hence in the lytic cycle.

Haematological abnormality was observed in one of the marmosets (245) inoculated with the *myc*-transfected cell line, but not in either the control animal (244) or the other animal (246) inoculated with the *myc*-transfected cell line. This suggests that the abnormality was not a direct consequence of introducing cells in which c-*myc* was constitutively expressed.

8. ATTEMPTS TO IDENTIFY THE ATYPICAL CIRCULATING MONONUCLEAR CELLS

8.1 Introduction

The presence of high numbers of morphologically atypical lymphocytes in the circulation of M245 led to speculation as to their identity, and the reasons for their appearance. The first possibility was that the atypical cells were those which had been inoculated into the animal, and which were persisting in the circulation. If this were the case, then one would expect to detect large numbers of EBNA positive cells in the peripheral blood. Secondly, they might be analogous to the atypical lymphocytes seen in infectious mononucleosis, which have been identified as CD8+ T cells (Crawford *et al.*, 1981a), and so should be detectable with T-cell specific antibodies. In an attempt to identify these atypical lymphocytes immunocytochemically, cells were stained with mAbs against human haemapoietic cell surface antigens. Secondly, cells from the peripheral blood were stained with human sera against EBNA to see if any circulating EBNA positive cells were present. Attempts were also made to establish spontaneously arising cell lines from the peripheral blood, and any

lines obtained analysed for the presence of EBNA and plasmid DNA sequences.

8.2 Immunocytochemical staining of peripheral blood mononuclear cells

Thin blood films prepared from the peripheral blood of the three marmosets were fixed in methanol and stained for the presence of haemopoietic cell surface antigens, using antibodies previously shown to react with marmoset lymphoid cells (Dr. J.A. Thomas, Imperial Cancer Research Fund, personal communication). Cells were stained with a human pan B-cell mAb (TO15, anti-CD22), a pan T-cell mAb (T11, anti-CD2) an anti-monocyte antibody (LeuM1), and an HLA DR alpha antibody (1B5). Details of the antibodies are given in chapter VI, section 18.1. Binding of antibody to antigen was visualised by incubating with rabbit anti-mouse Ig, followed by peroxidase anti-peroxidase and development with DAB. Unfortunately none of the antibodies used, with the exception of the anti-HLA DR alpha antibody, recognised any of the marmoset mononuclear cells, and thus it was not possible to distinguish lymphocyte and monocyte populations or identify the atypical cells. The anti-HLA DR alpha antibody recognised the majority of mononuclear cells, but, since the HLA DR molecules are present on both B and activated T cells, was of no value in identifying subsets of cells. It is not known why these antibodies failed to react with the marmoset cells, when previous studies have described them doing so. Technical shortcomings are unlikely to be the explanation since the 1B5 antibody clearly stained cells. Similar results were obtained when mononuclear cells were separated on centrifugation through a ficoll gradient, fixed in methanol and stained. In addition these experiments have been repeated using fresh samples of blood by Dr J.A. Thomas (Imperial Cancer Research Fund, London, UK) with similar results.

8.3 Detection of EBNA in peripheral blood mononuclear cells

To examine whether EBNA positive, and hence EBV infected, cells could be identified in the peripheral blood cytospin slide preparations of ficoll separated PBMs from marmosets 244 and 245 were made. These slides were stained using anti-complement IF (ACIF) to detect the presence of EBNA. Cells were stained with EBNA positive (JAT) and negative (WDA) human sera. As a control, cells from the EBV positive cell line, Raji, were stained with JAT and WDA. Despite examining PBM at day 84 when levels of atypical lymphocytes were high (25%) in marmoset 245, no EBNA positive cells were detected. Similarly, no positive cells were detected in the peripheral blood of the control marmoset (244).

Nuclear fluorescence was seen in Raji cells stained with JAT but not in cells stained with WDA.

8.4 Spontaneous cell lines

An attempt was made to establish spontaneously arising cell lines from the peripheral blood of marmosets 244 and 245. 10^6 ficoll-separated PBM from each marmoset, taken 111 days after inoculation, were cultured in the presence of cyclosporin A (1μg/ml, Sandoz) for 4 months. No proliferating cell lines were established, however a number of viable adherent and non-adherent cells were still present after 4 months in culture. There was no evidence that these cells were proliferating. These cells were not lymphoblastoid in morphology; those in suspension were large and regular in shape, and those cell adhering to the plastic tissue culture flask were dendritic in appearance suggesting that these cells were monocytes and macrophages, which are known to persist for a considerable length of time in culture (I. Reilly, Hammersmith Hospital, personal communication).

Cytospin slide preparations were made from these cells and stained for the presence of EBNA, by ACIF, using EBNA positve (RT) and negative (MJ) human sera. Staining with RT revealed nuclear fluorescence in all of the cells. However this staining proved non-specific as similar nuclear fluorescence was seen when the cells were stained with the EBNA negative serum. This non-specific staining may be a consequence of complement binding to complement receptors on the surface of monocytes. A similar phenomenon has been observed with human monocytes using the ACIF technique (Crawford, 1976).

Although the immunocytochemical data was inconclusive, the morphological data suggest that the cells persisting in culture were not EBV-immortalized lymphoblastoid cells which would normally be expected to proliferate, but were possibly macrophages or monocytes.

8.5 Summary

It was not possible to identify the atypical mononuclear cells in the circulation of marmoset 245, due to non-reactivity of B, T and monocyte specific antibodies used. It does appear clear, however, that they are not EBNA positive cells, and hence are not derived from the cells inoculated into the animal.

CHAPTER IV

DISCUSSION

1. GROWTH CHARACTERISTICS OF CELLS EXPRESSING A CONSTITUTIVELY ACTIVATED C-*MYC* GENE

1.1 Growth factor requirements

In normal growth medium *myc*-transfected human (IB4) and marmoset (M245) lymphoblastoid cell lines (LCL) reached higher cell densities than their respective control cell lines. However, the doubling times of these cell lines were not significantly shorter than the controls suggesting that, in EBV immortalised cells, constitutive expression of c-*myc* does not shorten significantly the time spent in G_1 as has been observed in other cell types (Karn *et al.*, 1989). The fact that the *myc*-transfected cells reached higher cell densities, supports the hypothesis that high level expression of the gene reduces growth factor requirements (Armelin *et al.*, 1984; Kaczmarek *et al.*, 1985; Sorrentino *et al.*, 1986). These observations are strongly supported by the enhanced proliferation of *myc*-transfected IB4 and M245 cell lines when cultured in reduced serum concentrations. A reduced serum dependency in EBV immortalised B cells transfected with the same c-*myc* containing plasmids has also been observed by Lombardi *et al* (1987). Thus, there is good evidence for activation of c-*myc* reducing the requirements for exogenous growth factors. There are a number of ways by which this might be achieved. Firstly, high level expression of c-*myc* might increase the responsiveness of cells to exogenous growth factors, perhaps as a consequence of increased affinity of growth factor receptors for their ligands. Alternatively activation of c-*myc* might stimulate production, or increased production, of autocrine growth factors. Two such putative growth factors of EBV immortalised B cells are CD23 and interleukin-6 (IL-6), both of which are reported as having autocrine growth factor properties *in vitro* (Swendeman and Thorley-Lawson, 1987; Tosato *et al.*, 1990).

The role of CD23 as an autocrine growth factor is disputed, as experiments using recombinant and purified native soluble CD23 failed to reveal any such activity (Uchibayashi *et al.*, 1989). The reasons for such discrepancies remains unclear, but Cairns and Gordon (1990) have recently demonstrated that the autocrine activity of recombinant soluble form of CD23 is extremely labile, with consistent auto-stimulatory effects not observed. No consistent changes in CD23 expression on the cell surface of *myc*-transfected cells were noted in this study. This does not, however rule out the possibility of either: i) increased synthesis and turnover of the molecule resulting in increased shedding of the putative autocrine growth factor, or ii) increased affinity of CD23 for its ligand. Neither

would be detected by simple analysis of cell surface expression. In addition expression of CD23 in cells cultured in low serum concentrations has not been examined.

IL-6 is produced by a wide variety of cells including fibroblasts, endothelium, keratinocytes and haemopoietic cells (reviewed by van Snick, 1990). IL-6 is known to stimulate growth of EBV-immortalised B cells (Tosato *et al.*, 1988), and recently has been described as an autocrine growth factor for EBV immortalised LCL (Tosato *et al.*, 1990). Interestingly IL-6 is only produced by LCLs cultured in low serum concentrations (Tosato *et al.*, 1990). In EBV immortalised LCL, transfection of a constitutively activated IL-6 gene results in a decreased serum dependency, and medium from IL-6 transfected cells can also stimulate growth of control cells (Scala *et al.*, 1990). The same authors also reported a tumorigenic phenotype for the IL-6 transfected cells, which grew in soft agar and produced tumours in nude mice. With respect to the serum dependency, constitutively expressed IL-6 and c-*myc* genes have similar effects on EBV immortalised B cells (Lombardi *et al.*, 1987; Scala *et al.*, 1990). Scala *et al.* (1990) also report preliminary unpublished data of *myc*-transfected LCLs producing high levels of autocrine growth factors, including IL-6. These data, if substantiated, supports the theory that the reduced serum dependency seen in these cells is a consequence of increased autocrine growth factor production.

The mechanism by which c-*myc* might induce expression of IL-6 is not known. The mechanism might be indirect, perhaps by inducing expression of other factors, such as IL-1, IL-3 and GM-CSF, known to induce expression of IL-6 (van Snick, 1990). Alternatively c-*myc* might bind directly to IL-6 control regions and activate expression of the gene. Several transcriptional enhancer sites have been identified immediately upstream of IL-6, including a c-*fos*-like serum response element (Ray *et al.*, 1988). Structural homologies led to the proposition that c-Myc might be a sequence specific DNA binding protein, similar to c-*fos*, although such activity has not yet been clearly demonstrated (chapter I, section 2.3). Whatever the role of CD23 and IL-6 in EBV immortalised LCL it is unlikely that either is important in the aetiology of BL since neither CD23 or IL-6 receptors are present on BL cells (Rooney *et al.*, 1986; Ling *et al.*, 1989; Taga *et al.*, 1987).

1.2 Cell-cell interactions

The decreased homotypic cell adhesion observed in *myc*-transfected IB4 cells has also been observed with other human LCLs transfected with the same plasmids (Lombardi *et al.*, 1987). The function of homotypic B-cell aggregation in EBV immortalised B cells is not clear, but it is a common feature of LCLs suggesting that it is important in some way in the growth of these cells. Gordon *et al.* (1985) reported that cell-cell contact in EBV immortalised B cells was essential for continued proliferation, and recently it has been shown that the intact membrane bound form of CD23 (45kDa) has stimulatory activity on activated B cells and LCL (Cairns and Gordon, 1990). Clearly cell-cell interaction would be required for this stimulation to occur. Infection of B cells by EBV results in increased expression of LFA-1 (Allday *et al.*, 1989), and upregulation of this molecule has been observed in EBV-negative BL cell lines transfected with LMP (Wang, D. *et al.*, 1988). However, expression of this molecule is not essential to the immortalisation process, since EBV immortalised LCLs can be established from the peripheral blood of patients suffering from leucocyte adhesion deficiency (LAD) (Krensky *et al.*, 1985). LAD is a congenital disease in which expression of the beta subunit of LFA-1, Mac-1 and p150,95 is impaired, resulting in greatly reduced (<0.2 - 5% of normal levels) expression of these molecules (Kishimoto *et al.*, 1987).

Adhesion between EBV immortalised lymphoblastoid cells has been shown to be mediated via LFA-1 and its ligand ICAM-1 (Mentzer *et al.*, 1985; Rothlein *et al.*, 1986). Thus the decreased aggregation of *myc*-transfected cells seen in this and previous studies (Lombardi *et al.*, 1987) is probably a consequence of decreased expression of LFA-1. ICAM-1 is not expressed on IB4 cells suggesting that the aggregation of IB4 cells is mediated via LFA-1 and another ligand such as ICAM-2. It was not possible to examine expression of this molecule as antibodies are not yet available (Dr D. Staunton, Harvard Medical School, Boston, USA, personal communication).

2. CELL SURFACE PHENOTYPE

2.1 Markers of B cell differentiation

Expression of a panel of B cell differentiation markers was examined using monoclonal antibodies. No significant differences were observed when *myc*-transfected and control IB4 cell lines were compared. The phenotype of the *myc*-transfected cell lines was similar to the control cell line, *i.e.* a typical LCL

expressing B cell activation markers such as CD23, CD30 and CDw77. Analysis of expression of these markers provided no evidence for constitutive expression of c-*myc* affecting the differentiation status of these cells.

2.2 Expression of HLA molecules.

High level expression of c-*myc* and N-*myc* has been shown to result in a down-regulation of HLA class I molecules and has been proposed as a general mechanism by which tumour cells evade immunosurveillance (Versteeg *et al.*, 1988; Bernards *et al.*, 1986). Down regulation of class I molecules has also been observed in adenovirus type 12 transformed cells. It was demonstrated that evasion of the cell mediated immune response was an important factor in the genesis of tumours following inoculation of these cells into immunocompetent syngeneic animals (Schrier *et al.*, 1983; Bernards *et al.*, 1983). Confirmation of these findings was provided by Tanaka *et al* (1985) who reported that the tumorigenic properties of these cell lines could be reversed by transfection of the relevant class I molecules driven by heterologous promoters. Down-regulation of class I molecules seems to be a feature of virus-induced transformation, since such regulation is not common in spontaneously occurring tumours (Bernards, 1987). There are two notable exceptions to this rule, namely small cell lung carcinoma and neuroblastoma involving amplified c-*myc* and N-*myc* genes respectively (Doyle *et al.*, 1985; Little *et al.*, 1983; Brodeur *et al.*, 1984; Bernards *et al.*, 1986). Interestingly amplification of N-*myc* in neuroblastoma does not occur until metastasis, and the degree of amplification correlates with disease progression (Brodeur *et al.*, 1984).

No change in expression of either HLA I or HLA DR was observed in these experiments, but whether down-regulation of specific loci would be masked by using a broadly reactive HLA class I monoclonal antibody is not clear. Further studies would be required using monospecific reagents to conclusively show that in EBV immortalised B cells constitutive expression of c-*myc* does not result in a decrease in expression of HLA class I molecules. The evidence of Masucci and co-workers (Masucci *et al.*, 1987; Torsteinsdottir *et al.*, 1988) suggests that the pan HLA I antibody, W6.32, would not detect selective down-regulation of specific HLA alleles. They examined HLA I expression in a panel of BL cell lines and described low level expression of one locus (HLA A11), as determined by staining with an antibody specific for a common determinant on A3 and A11. However, staining with W6.32 did not reveal any overall decrease in HLA I. It was proposed that failure to express this molecule could contribute to

development of the tumour by preventing lysis by HLA I restricted autologous cytotoxic T lymphocytes (CTL).

2.3 Cell adhesion molecules

It has also been proposed that down-regulation or failure to express cell adhesion molecules involved in interactions between B cells and CTL might be an important step in tumour development (Clayberger *et al.*, 1987; Gregory *et al.*, 1988b). As discussed earlier the two pathways involved in B cell-CTL interactions are LFA-1-ICAM-1 and LFA-3-CD2 (chapter I, section 3.3). A consistent decrease in expression of LFA-1 was observed on *myc*-transfected IB4 and CB33 cells relative to control cells (discussed below). As discussed earlier (section 1.2) ICAM-1, the best characterised ligand for LFA-1, was not expressed on *myc*-transfected or control IB4 cells, however no difference in expression of ICAM-1 was observed on *myc*-transfected CB33 cells relative to control cells. No difference in expression of the other cell adhesion molecule expressed on EBV infected B cells - LFA-3 - was observed when *myc*-transfected and control IB4 and CB33 cells were compared.

3. EXPRESSION OF LFA-1

3.1 Regulation of LFA-1 expression

A consistent decrease in expression of LFA-1 on the cell surface was observed in all human *myc*-transfected LCLs examined, which led to the question of the mechanism by which LFA-1 is down regulated in these cell lines. Data from this study has indicated that the decreased expression of LFA-1 seen on the surface of cells constitutively expressing c-*myc* is a consequence of altered alpha chain gene expression. Preliminary data also indicates that the point at which down-regulation occurs is at initiation of transcription, although additional involvement of post-transcriptional mechanisms such as mRNA stability and transport cannot be ruled out. These data, however, are substantiated by the findings of another laboratory (data presented by Dr R Dalla-Favera, 15th International Cancer Congress, Hamburg, August 1990).

It is possible that c-*myc* might act directly to suppress transcription of the alpha chain gene, perhaps in a manner analogous to its own autosuppression where suppression of c-*myc* transcription is directly proportional to the level of c-Myc protein (Penn *et al.*, 1990a). Attempts were made in this study to address this

possibility by using c-*myc* deletion mutants to map regions, if any, involved in suppression of LFA-1 alpha chain transcription. Unfortunately this approach has so far proved unsuccessful, but warrants further attention. Another approach would be to examine any potential influence of c-Myc on LFA-1 alpha enhancer regions. However, this approach is hampered, at present, by the lack of data on the regulatory regions upstream of the LFA-1 alpha and beta chain genes.

A problem with the hypothesis of c-*myc* directly regulating LFA-1 expression is the paradox of some BL cell lines, with an activated c-*myc* gene, expressing high levels of LFA-1 (Billaud *et al.*, 1990). If c-*myc* were acting on LFA-1 expression one might expect all BL cell lines to express low or absent levels of LFA-1. However, as Penn and colleagues demonstrated, autosuppression of c-*myc* transcription is directly correlated to the level of c-Myc protein and a threshold operates, below which autosuppression does not occur (Penn *et al.*, 1990a). It is possible that a similar mechanism might operate with regulation of LFA-1. It would be interesting to attempt to correlate levels of c-Myc with levels of LFA-1.

There have been a few reports describing c-Myc apparently regulating expression of other cellular genes. C-Myc has been described activating heat shock protein promoters (Kingston *et al.*, 1984) and suppressing activity of the metallothionein I promoter (Kaddurah-Daouk *et al.*, 1987). Also post-transcriptional modification of two cellular genes mr1 and mr2 by Myc has been observed (Prendergast and Cole, 1989). Down-regulation of HLA I (Versteeg *et al.*, 1988), and histone H1 (Cheng *et al.*, 1989) by c-*myc* has also been observed but the mechanisms involved are not characterised. Down-regulation of HLA-I by another member of the *myc* family, N-*myc,* (Bernards *et al.*, 1986) is thought to occur as a result of impairing binding of transcription factors to the HLA-I enhancer region (Lenardo *et al.*, 1989).

The mechanism by which LFA-1 is down-regulated may well be indirect. One possibility is that c-Myc might have regions homologous to DNA binding regions of LFA-1 alpha transcription factors but lack the necessary regions to transactivate LFA-1. High level expression of c-*myc* might compete preferentially for these DNA binding sites, preventing activation of the gene. Alternatively c-Myc might form complexes with LFA-1 alpha chain transcription factors and prevent their binding to the alpha chain enhancer regions.

It could be argued that the down-regulation of LFA-1 might be a consequence of a change in the differentiation status of *myc*-transfected cells. No change in the differentiation status of *myc*-transfected B cells, relative to control cells, was noted (section 2.1), but it is possible that the antibodies used do not detect subtle changes in differentiation. Whilst this possibility cannot be completely ruled out, there is no evidence for constitutive expression of c-*myc* inducing differentiation, indeed most of the evidence suggests that the effect of high level expression of c-*myc* prevents further differentiation occurring (chapter 1, section 2.3). In addition, expression of LFA-1 has been shown to increase during B cell differentiation, rather than decrease (Kansas and Dailey, 1989). It is also difficult to argue that decreased expression of LFA-1 is a consequence of c-*myc* inducing a more activated phenotype since activation of B-cells has been to increase rather than decrease expression of LFA-1 (Wang, D., *et al.*, 1988b; Allday *et al* 1989).

3.2 LFA-1 and Burkitt's lymphoma

The importance of LFA-1, LFA-3 and ICAM-1 in interactions of B cells with EBV specific cytotoxic T lymphocytes (CTL) was clearly demonstrated by Gregory *et al.*(1988b) who found that BL cells lacking these molecules could not form conjugates with CTL, an essential prerequisite for lysis to occur (Springer *et al.*, 1987). In addition these cells were also resistant to lysis by EBV specific CTL. The data of Gregory *et al* (1988b) support the proposition that down-regulation of these molecules in BL might contribute to evasion of the cell mediated response to EBV.

Antibody blocking experiments have shown that LFA-1:ligand interaction between CTL and target cells is unidirectional, with LFA-1 on the T cell and ligand on the target (Krensky *et al.*, 1983; Krensky *et al.*, 1985). However, the importance of LFA-1 on the target cell is clearly demonstrated in LFA-1 deficient individuals. Krensky *et al.* (1985) demonstrated that interactions between CTL and target can be mediated via LFA-1 on the target and ligand on the CTL. In addition, lysis of LFA-1 negative target cells by CTL from LAD patients is markedly less efficient than lysis of LFA-1 positive target cells (Mentzer *et al.*, 1986). These data clearly indicate that expression of LFA-1 on the target cells is functionally important in CTL interactions. The interaction between helper T cells (T_H) and B cells has also been reported as involving LFA-1 on the T cell and ligand (ICAM-1) on the B cell (Mazerolles *et al.*, 1988).

However, similar work looking at the interaction between T_H and B cells in mice have revealed some interesting results. Incubation of memory B cells with anti-LFA-1 monoclonal antibody did not inhibit conjugation with T_H, but incubation of virgin B cells with the same antibody did inhibit such conjugation (Sanders *et al.*, 1987). This suggests that the role of LFA-1 on the B cell may be differentiation dependent. Further evidence for the importance of LFA-1 on target cells comes from studies which show that EBV immortalised B cell lines from LFA-1 deficient individuals are poor stimulators in mixed lymphocyte response (MLR) assays (Krensky *et al.*, 1985; Clayberger *et al.*, 1987).

In view of this evidence suggesting an important role for LFA-1 on target cells it would be interesting to examine the response of T cells to the *myc*-transfected IB4 cell lines. Preliminary experiments in our laboratory, done in collaboration with Ms K. Burman, indicate that *myc*-transfected cells are no more resistant to lysis by autologous EBV specific CTL than control cell lines. Similar results were found with *myc*-transfected and control CB33 cells. Further work is required to confirm these findings, but they are perhaps not surprising since the down-regulation of LFA-1 in the *myc*-transfected cells is not complete, and relatively low levels of LFA-1 have been shown to be functionally significant (Springer *et al.*, 1987). This is illustrated by comparing the CTL responses of patients with moderate LAD, expressing approximately 5% of normal levels of LFA-1, and patients with severe LAD, in whom expression of LFA-1 is less than 0.2% of that found in normal individuals. Patients with severe LAD have CTL responses 80-90% lower than those seen in normal patients, whereas patients with moderate LAD have CTL responses 50% lower than normal (Krensky *et al.*, 1985). Thus, since expression of LFA-1 in *myc*-transfected cells is 50% of that in control cells, conjugation with CTL may not be significantly impaired.

Despite the theories for BL cells evading immunosurveillance it is not clear that such a mechanism is in any way important in the development of BL. There is, as yet, no evidence for the sole latency associated antigen expressed in BL cells (EBNA-1) acting as a target for EBV specific CTL. If it does not, then evasion of EBV specific cytotoxic T cells could be considered an irrelevant concept, since none of the EBV antigens known to act as targets for EBV specific CTL are expressed in BL cells (chapter I, section 4.8). Expression of the cell adhesion molecules may, however, be important in interactions between BL cells and other cells of the immune system. For example, LFA-1 is known to be important in natural killer (NK) cell activity (Kohl *et al.*, 1984). The available data, however, suggests that interactions between NK and target cell is independent

of LFA-1 expression on the target cell (Schmidt *et al.*, 1985).

Apart from the question of evading the immune system there are other ways in which LFA-1 might be involved in the development of a malignant phenotype. There is some evidence implicating LFA-1 in signal transduction. Van Noesel *et al* (1988) examined the proliferative response of T cells to a panel of monoclonal antibodies against the alpha and beta subunits of LFA-1. All anti-alpha chain antibodies enhanced proliferation whereas five out of six anti-beta chain antibodies inhibited proliferation. Thus LFA-1 may act not only to facilitate adhesion between T and B cells, but may also be involved directly in inhibiting or stimulating proliferation of T cells. Whether such properties are found in other cell types expressing LFA-1 is not known.

One of the classical characteristics of transformed fibroblast and epithelial cells is reduced cell-substrate interactions, resulting in anchorage independent growth (MacPherson and Montagnier, 1964). Plantefaber and Hynes (1989) demonstrated that decreased adhesion of Rous sarcoma virus and *ras* transformed cells to substrate is a consequence of decreased expression of the fibronectin receptor. In common with LFA-1, the fibronectin receptor is an integrin molecule and down-regulation of integrin molecules may be a characteristic of transformed cells. Recently, transfection of transformed epithelial cells with cDNAs encoding the fibronectin receptor has been shown to result in a reversion of the tumorigenic phenotype, both in terms of growth in soft agar and tumour production in nude mice (Giancotti and Ruoslahti, 1990). The authors concluded that interaction between the fibronectin receptor and the extracellular matrix plays a role in control of cell proliferation. Whilst caution should be exercised in extrapolating between different integrin molecules and different cell types, it is tempting to speculate that LFA-1 might be involved in a similar mechanism. If so, down-regulation of this molecule could play an important role in development of the malignant phenotype. It would be interesting to transfect the *myc*-transfected lymphoblastoid cells with constitutively active, or inducible, LFA-1 alpha and beta subunit cDNAs and compare growth characteristics relative to *myc*-transfected cells.

The down-regulation of another cell adhesion molecule, N-CAM, by a member of the *myc* family - N-*myc* - has also been reported (Akeson and Bernards, 1990). Similar to the results of this study the point at which altered regulation of N-CAM occurred was thought to be intiation of transcription. In human neuroblastoma amplification of N-*myc* does not occur until metastasis, and the

degree of amplification correlates with disease progression (Brodeur *et al.*, 1984). What is not known is whether amplification of N-*myc* is a consequence of metastasis or the cause, but if overexpression of N-*myc* does result in down-regulation of cell adhesion molecules it is conceivable that amplification of N-*myc* is the event which triggers metastasis.

LFA-1 might also play a role in formation of germinal centres and interactions between B cells and secondary lymphoid tissue including follicular dendritic cells (FDC) of the germinal centres. ICAM-1 is known to be expressed on FDC and germinal centre B cells (Dustin *et al.*, 1986). LFA-1 is also expressed on germinal centre B cells (Smith and Thomas, 1990), so it is not inconceivable that LFA-1 might be important in such interactions. A recent report describing the blocking of interactions between lymphoid cells and FDC using anti-LFA-1 monoclonal antibodies suggests such interactions may occur *in vivo* (Louis *et al.*, 1989). The paradox of BL cells having a germinal centre B cell phenotype yet tumours rarely being observed in lymphoid tissue might be explained by lack of expression of LFA-1. If LFA-1 acts as a homing receptor for B cells, then down regulation of the molecule might prevent formation of the tumour in the germinal centre. Support for this theory is provided by the data of Stauder *et al* (1989) who compared LFA-1 expression of various B cell tumours, and found that those localised to lymphoid follicles expressed LFA-1, whereas extranodal malignancies generally do not. Interestingly studies of LFA-1 expression on non-Hodgkin's lymphoma have revealed that those considered high grade malignancies tend to express low or absent levels of LFA-1 whilst those that are low grade express relatively high levels of LFA-1 (Inghirami *et al.*, 1988; Stauder *et al.*, 1989).

Another B cell integrin molecule VLA-4 has also been demonstrated mediating adhesion between B cells and germinal centre FDC via interaction with the INCAM-110 cell adhesion molecule expressed on FDC (Freedman *et al.*, 1990). It would be interesting to examine expression of this molecule on BL and *myc*-transfected cell lines.

4. VIRAL GENE EXPRESSION

4.1 Latency associated antigens

No differences in levels of expression of the EBV latency associated antigens, EBNAs 1-6 and LMP, were observed when *myc*-transfected and control IB4 cell lines were compared by immunoblotting. This suggests that the restricted pattern of EBV gene expression seen in BL cells, where only EBNA-1 is expressed, is not a direct consequence of activation of c-*myc*. This is supported by the observation that BL cell lines, cultured *in vitro*, tend to undergo drift towards a lymphoblastoid phenotype expressing all of the EBNAs and LMP (Rowe M., *et al.*, 1987b).

A link between expression of LMP and expression of LFA-1 has also been reported. Transfection of an EBV negative BL cell line with LMP results in a more activated phenotype, including upregulation of LFA-1, LFA-3 and ICAM-1 (Wang *D.*, *et al.*, 1988b). No difference in levels of LMP expression was observed in *myc*-transfected cells relative to control cells, indicating that the altered expression of LFA-1 was not a consequence of decreased LMP expression.

Although no overall difference in levels of LMP expression was observed in *myc*-transfected IB4 cells, differences in location of LMP within the cell were observed using indirect immunofluorescence. LMP in *myc*-transfected IB4 cells was localised to patches, whereas in the control cells the staining was more diffuse. In CB33 cells no such differences were observed between control and *myc*-transfected cells, with both cell lines demonstrating a diffuse staining pattern similar to the IB4-HEBo(30/3) control cell line. The different staining pattern seen in IB4-myc123(24/3) might be explained by the fact that vimentin, a component of the cytoskeleton with which LMP is known to associate (Liebowitz *et al.*, 1987), is expressed only at very low levels in *myc*-transfected IB4 cells. By contrast, in IB4-HEBo(30/3) and both *myc*-transfected and control CB33 cells vimentin is expressed normally. Thus the patchy LMP staining may well be a consequence of disrupted vimentin expression. The reason for decreased expression of vimentin in *myc*-transfected IB4 cells, but not CB33 cells, is not known. Vimentin has been reported as being absent from BL cell lines but expressed at high levels in LCL (Dellagi *et al.*, 1984). The results of this study generally support this observation. However, in contrast to the findings of Dellagi *et al* (1984), cells of the BL cell line, Raji, were shown to express vimentin, albeit at low level and with unusual patchy distribution.

4.2 Expression of lytic cycle antigens

Experiments looking at expression of EBV lytic cycle antigens, EA and VCA, in IB4 transfectants revealed some potentially interesting data. LCLs derived from cord blood lymphocytes, such as IB4 (King *et al.*, 1980), tend to express lytic cycle antigens in only a very small percentage of cells compared to LCL derived from adult lymphocytes (Crawford *et al.*, 1979). IB4 is no exception, with only 0.004% and 0.002% of cells from the parental and pHEBoSV-transfected control cell lines respectively expressing EA. In contrast to most other LCL however, only EA was expressed with no VCA being detected. EA was not detected in either of the *myc*-transfected IB4 cell lines. The reason why EA is expressed, but not VCA, is not known, but might be a reflection of the fact that the viral genome is thought to be integrated with no dectectable viral episomes (Henderson, *et al.*, 1983; Ms M. Doyle, London School of Hygiene and Tropical Medicine, London, personal communication; Dr E Hurley, Tufts University, Boston, USA, personal communication).

Treatment of cells with TPA, known to induce lytic cycle antigens (Zur Hausen *et al.*, 1978), resulted in three to four times as many control IB4-HEBo(30/3) cells expressing EA as IB4-myc123(24/3) cells. These data suggest that constitutive expression of c-*myc* in EBV immortalised LCL prevents cells entering the lytic cycle. TPA is known to induce differentiation of B cells (Totterman *et al.*, 1980) and expression of lytic cycle antigens has been linked to terminal differentiation of lymphoblastoid cells (Crawford and Ando, 1987). This suggests that constitutive expression of c-*myc* in IB4 cells is preventing terminal differentiation and hence expression of lytic cycle antigens. This would be in line with observations of high level expression of c-*myc* preventing differentiation in a number of other cell types (Coppola and Cole, 1986; Prochownik *et al.*, 1986; Freytag, 1988).

5. THE ROLE OF C-*MYC* IN TUMOUR DEVELOPMENT

These experiments have demonstrated that whilst high level constitutive expression of c-*myc* results in altered growth characteristics and cell surface phenotype, it does not necessarily result in a tumorigenic phenotype, as determined by the classically used assays of growth in soft agar and tumour production in nude mice. As described in the introduction the role of c-*myc* as a

'single-hit' oncogene is controversial. Whilst the oncogenic potential of v-*myc* has been demonstrated *in vitro* and *in vivo*, the majority of work with c-*myc*, including transgenic mice experiments, suggests that activation of c-*myc* alone is insufficient for malignant transformation (chapter I, section 2.4). In experiments similar to those described in this thesis, Lombardi *et al* (1987) concluded that constitutive expression of c-*myc* in EBV immortalised B cells was sufficient to induce a tumorigenic phenotype, as indicated by growth in soft agar and production of tumours in nude mice. Their data is in direct contrast to the findings of this study which shows no evidence, *in vitro* or *in vivo*, for constitutive c-*myc* expression in EBV immortalised human, or marmoset, B cells resulting in a tumorigenic phenotype. Whilst there is no clear explanation for the discrepancy between the two studies, there are a number of possible explanations.

Firstly, it may be that, in the cell lines used by Lombardi *et al* (1987), other genetic events had occurred resulting in activation of another oncogene, such as *ras*, which could act in synergy with c-*myc* to induce a malignant phenotype (Land *et al.*, 1983, 1986). Whilst this explanation is theoretically possible, it is difficult to see how this could occur simultaneously in all of the four *myc*-transfected cell lines examined by Lombardi *et al* (1987), but not in the cell lines used in this study. The presence of pre-existing activated oncogenes is also unlikely since the cell lines used by Lombardi *et al* (1987) were derived from cell stocks frozen after less than ten passages in culture.

An alternative explanation is that there is a correlation between tumorigenic potential of these cell lines and levels of c-Myc protein, as was suggested by Lombardi *et al* (1987). This notion is supported by the fact that Raji cells, which were tumorigenic in one set of experiments, had significantly higher levels of c-Myc than the non-tumorigenic *myc*-transfected IB4 and M245 cell lines. However, when the levels of c-Myc protein in one of Lombardi's tumorigenic cell lines (CB33-myc23) was compared to the levels of c-Myc in IB4 cells transfected with the same plasmid, no significant difference was observed.

Potentially, the cell lines used in this study represent a population of cells more resistant to malignant transformation by c-*myc*, perhaps as a result of a difference in the differentiation status of the cells. This would be consistent with the Klein theory that oncogenes can only exert their tumorigenic effects during relatively narrow "differentiation windows" (Klein and Klein, 1986). Whilst this theory cannot be excluded, it seems unlikely that both IB4 and

M245 LCLs would be resistant to malignant transformation, whilst the two cell lines used by Lombardi *et al* (1987) were susceptible.

Finally, it may be that the differences between the data in this study and that of Lombardi *et al* (1987) reflect differences in experimental methods, or variations in susceptibility of different strains of nude mice to tumour development. With this possible explanation in mind, the experiments involving the common marmosets were devised. It was felt that the common marmoset represented a good model in which to study the tumorigenic potential of EBV immortalised lymphoblastoid cells constitutively expressing c-*myc* (chapter III, section 1). Compared to the somewhat artificial system of nude mice, the common marmoset mimics far more closely the *in vivo* environment in which tumours develop. Nude mice have severe cell mediated immune deficiencies, yet in BL, whilst transient immune suppression may occur during acute malaria (Whittle *et al.*, 1984) T cell dysfunction is not pronounced (Rooney *et al.*, 1985b). This is supported by the fact that the EBV associated lymphoproliferations seen in severely immunocompromised individuals have not been observed in children from areas where malaria and BL are prevalent (Lenoir and Bornkamm, 1979). Furthermore, inoculation of common marmosets with EBV does not result in formation of tumours as is the case with cotton-top marmosets (Wedderburn *et al.*, 1984; Shope *et al.*, 1973; Epstein and Morgan, 1986). Over a six month period following inoculation of either a control or *myc*-transfected marmoset LCL, none of the marmosets examined in the present study developed tumours, and they remained in apparent good health. The appearance of abnormal mononuclear cells in one of the two marmosets inoculated with the *myc*-transfected cell line raised the possibility that these cells were derived from those inoculated into the animal. It was not possible, by immunocytochemical means, to establish the identity of the atypical mononuclear cells in the circulation of this marmoset. Indeed, as noted earlier (chapter III, section 8.2) it proved difficult to identify any lymphocyte subsets from the peripheral blood of the three marmosets. This was despite the fact that the antibodies used had been shown to react with antigens on marmoset lymphoid tissue (Dr J.A. Thomas, Imperial Cancer Research Fund, London, unpublished observations). Since EBNA-positive cells could not be identified in the peripheral blood of marmoset 245 at a stage when these cells formed a high proportion of the total mononuclear cell count, it is highly unlikely that these cells were derived from the inoculated M245-myc123 cell line. In addition M246, also inoculated with M245-myc123, did not have a high proportion of atypical cells at any stage following inoculation. This suggests that the atypical cells

were not a consequence of inoculation of M245-myc123 cells, but perhaps were an indication of some unrelated haematological condition. Since the completion of this study, cytogenetic studies, carried out by Dr I. Reilly and Dr J. Bungey (Department of Haematology, Hammersmith Hospital, London) have revealed the presence of a reciprocal chromosome translocation in PBM from marmoset 245 stimulated with the pokeweed B cell mitogen. This translocation, involving chromosomes 2 and 9, was detected in two out of nine metaphases examined. It was not seen in cells stimulated with the T cell mitogen phytohaemagglutinin, suggesting that the cells with the translocation might be B cells. Translocations were not seen in cells from marmosets 244 and 246, or in the M245 and M245-myc123 cell lines inoculated into the animals. It is not known whether the translocation was present prior to the start of the experiment, nor whether the presence of large numbers of atypical cells are a consequence of this translocation. Similarly, it is not known which marmoset genes are located on these chromosomes, nor is it known whether such translocations are common in these animals. However, it seems unlikely that this translocation is a consequence of inoculation with M245-myc123 cells.

The absence of tumours or EBNA-positive circulating B cells in any of the animals suggests that the inoculated cells were eliminated by the cell mediated immune system, presumably as a consequence of the cells expressing the EBV genes recognised by CTL. This implies that constitutive expression of c-*myc* is insufficient to induce a phenotype capable of evading immunosurveillance. It would be interesting to attempt further experiments in which marmosets inoculated with *myc*-transfected or control cell lines were concurrently; i) immunosuppressed with cyclosporin A or, ii) infected with malaria. One could speculate that treatment with cyclosporin A might result in the appearance of tumours similar to the EBV associated tumours seen in humans with profound T cell dysfunction (chapter I, section 5.7.2). These tumours might well arise both in animals inoculated with *the myc*-transfected cell line and the control cell line. If, as seems likely, the T cell dysfunction caused by malaria infection is not sustained following acute infection (Whittle *et al.*, 1984), one would not expect such tumours to arise following malaria infection of the marmoset.

There is evidence supporting the findings of this study, with respect to the tumour producing potential of *myc*-transfected LCLs in nude mice. Wolf *et al* (1990) fused tumorigenic BL cells with non-tumorigenic lymphoblastoid cells derived from the same patient and examined the tumorigenic potential of these hybrid cells *in vivo*. Despite the presence of high levels of c-*myc* transcription,

the hybrid cells were non-tumorigenic in nude mice. They concluded that the lymphoblastoid cell contributes an, as yet unidentified, tumour suppressing function. This tumour suppressing function could be viral or cellular in origin, but is unlikely to be provided by expression of EBNA 2-6 or LMP since monoclonal tumours expressing the full range of EBV latency associated antigens have been shown to arise in patients with severe T cell dysfunction (chapter I, section 5.7.2).

These results have implications in the development of BL. Both of the proposed models for development of BL assume that the presence of EBV and the activation of c-*myc* are sufficient for malignant transformation to occur (Klein, 1979a, Lenoir and Bornkamm, 1987). The data from this study, and that of Wolf *et al* (1990) suggest that this may not be the case - a further unidentified factor may be required. This factor might simply be the selective down regulation of certain EBV genes, or it might involve activation of other oncogenes. Of course it is not possible to exclude the possibility that the results of this study merely reflect the use of an *in vitro* EBV infected B cell as a model for the effects of constitutive expression of c-*myc*. The *in vitro* cell clearly differs from the *in vivo* cell in terms of differentiation and viral gene expression. It is entirely possible that activation of c-*myc* and infection of the precursor BL cell with EBV *in vivo* is sufficient for malignant transformation. The ideal cell type on which to perform these experiments would be the putative BL precursor, the germinal centre B cell (Lennert and Mohri, 1978; Gregory *et al.*, 1987). Transfection of an activated c-*myc* gene into an *in vitro* EBV infected germinal centre B cell, however, would clearly be no more relevant since infection of these cells *in vitro* results in a lymphoblastoid phenotype with expression of all of the latency associated antigens (Gregory *et al.*, 1988a). A potentially more useful approach would be to co-transfect with plasmids encoding EBNA-1 and c-*myc*. Alternatively, an EBV based vector, such as the one devised by Hammerschmidt and Sugden (1989), could used to introduce EBNA-1 and c-*myc* into germinal centre B cells.

6. THE ROLE OF C-*MYC* AND EBV IN BL

There is no evidence that BL patients have impaired EBV specific CTL responses (Rooney *et al.*, 1985b) and clearly the available evidence suggests that expression of EBNAs 2, 3, 6 and LMP is incompatible with continued cell

survival *in vivo*, since these cells would presumably be eliminated by the cell mediated immune response to EBV. This suggests that the viral genes that are thought to be involved in the *in vitro* immortalisation of B cells by EBV (EBNA-2 and LMP) play no role in the development of BL.

The presence of EBV specific CTL exerts a strong selective pressure for the survival of EBV infected B cells in which expression of EBNA 2-6 and LMP is down-regulated. This is illustrated by the fact that B cells expressing EBNAs 2-6 and LMP have not been identified in normal healthy individuals (Ling *et al.*, 1989), and are only observed *in vivo* in patients with profound T cell dysfunction, such as those undergoing immunosuppressive therapy following organ transplantation (Young *et al.*, 1989; Thomas *et al.*, 1990b; chapter I, section 5.7.2).

It could be argued that EBNA-2 and/or LMP might play a role in the development of BL by being transiently expressed at certain crucial stages in tumour development. Their role might be to promote proliferation of EBV infected pre-B cells, thus increasing the chances of a chromosome translocation involving c-*myc* occurring. This still leaves the question of how cells expressing EBNA-2 and LMP evade EBV specific CTL. One possibility is that some or all of the stages in BL development occur in immunologically privileged sites, where cytotoxic T cells are excluded (Streilein and Wegmann, 1987). If this were the case, however, one might expect the tumours to occur in such sites. Whilst BL is seen in such sites, for example the testes, they are not common sites of presentation (chapter I, section 4.2). Alternatively, early stages of tumour development might occur during acute malaria when EBV-specific CTL responses are known to be depressed (Whittle *et al.*, 1984). Activation of c-*myc*, and subsequent down-regulation of cell adhesion molecules and/or HLA class I molecules would then enable the cell to evade the cell mediated immune response. Obviously if this were to occur, then the selective pressure to down-regulate EBNA 2-6 and LMP would be removed, in which case one might expect BL cells to express these viral antigens.

The hypothesis presented above cannot be excluded, but a conceptually simpler hypothesis involves EBNA-1 and c-*myc* cooperating to induce a malignant phenotype. The normal sequence of events following EBV infection of a B cell, *in vivo,* is not known. Klein (1989) has proposed that the latently infected B cell is a small resting B cell expressing EBNA-1, but not EBNA 2-6 and LMP. It is possible that the normal course of events involves progression from a self-

renewing, 'latently infected', EBNA-1 positive B cell to an EBNA-1 negative, terminally differentiating, virus-producing B cell, without expression of the other latency associated antigens. A translocation and subsequent activation of c-*myc* in such a cell might lead to uncontrolled proliferation of a cell unable to progress along the differentiation pathway, produce virus and die. Alternatively, EBV infection of a B-cell containing a translocated c-*myc* gene, might result in a similar phenotype. As mentioned in section 5, the selective down-regulation of viral genes with putative tumour suppressor activity may be an important additional factor in the development of BL.

How might EBNA-1 and c-*myc* cooperate to induce a malignant phenotype? EBNA-1 is thought to promote viral DNA replication by binding to the episomal origin of replication (OriP) (Yates, *et al.*, 1984, 1985; Sugden and Warren, 1989). Whilst interaction between EBNA-1 and host cell DNA has not been demonstrated, it is tempting to speculate that EBNA-1 could promote host cell DNA replication. In this respect EBNA-1 would complement c-*myc*, since high level expression of c-*myc* appears to increase sensitivity to growth factors and prevent exit from the cell cycle, but not directly promote DNA synthesis (Armelin *et al.*, 1984; Kaczmarek *et al.*, 1985). Support for the role of EBNA-1 as a potential oncogene is provided by experiments in which an EBNA-1 transgenic mouse developed a lymphoma (Dr J. Wilson, Princeton University, USA, personal communication). In this hypothesis the role of malaria would be to induce polyclonal B cell activation, increasing the chances of a chromosomal translocation involving c-*myc*.

CHAPTER V

MATERIALS AND REAGENTS

1. SUPPLIERS

Amersham International plc, Amersham, Bucks, UK.
Beckman Instruments, Palo Alto, California, USA.
BDH Ltd., Poole, Dorset, UK.
Biological Industries, Kibbutz Beth Haemek, Israel.
BioPolymers Inc., Farmington, Conneticut, USA.
Bio-Rad Laboratories, Richmond, California, USA.
Boehringer Mannheim UK, Lewes, East Sussex.
Calbiochem, Novabiochem (UK) Ltd., Nottingham, UK.
Camlab Ltd., Cambridge, UK.
Coulter Electronics, Luton, Beds, UK.
Dako Ltd, High Wycombe, Bucks, UK.
Difco Laboratories, Detroit, Michigan, USA.
Dow Chemical Co., Bishop's Stortford, Herts, UK.
DuPont (UK) Ltd, Stevenage, Herts, UK.
Dynatech, Billingshirst, Sussex, UK.
Falcon, Becton Dickinson UK Ltd, Oxford, UK.
Flow Laboratories Ltd, Irvine, Ayrshire, UK.
Flowgen Instruments, Sittingbourne, Kent, UK.
Fluka Chemie AG, Glossop, Derbyshire, UK.
FMC BioProducts, Flowgen Instruments, Sittingbourne, Kent, UK.
Gibco BRL Ltd, Uxbridge, Middlesex, UK.
Grant Instruments, Cambridge, UK.
Gull Laboratories, Salt Lake City, Utah, USA.
Hendley, Loughton, Essex, UK.
ICN Biomedicals Inc., High Wycombe, Bucks, UK.
IQ [Bio] Ltd., Cambridge, UK.
LEEC Ltd, Nottingham, UK.
Leitz, Wetzlar, FRG.
MSE, FSA Laboratory Supplies, Leicester, UK.
New England Biolabs, CP Laboratories, Bishop's Stortford, Herts, UK.
Nunc, Gibco BRL Ltd, Uxbridge, Middlesex, UK.
Oxoid, Unipath Ltd., Bedford, UK.
Painse and Byrne, Greenford, Middlesex, UK.
Pharmacia LKB Biotechnology, Uppsala, Sweden.
Schleicher and Schuell, Dassel, FRG.
Scotlab, Bellshill, Scotland.
Shandon Scientific Ltd, Runcorn, Cheshire, UK.

Sigma Chemical Co., Poole, Dorset, UK.
Skatron AS, Tranby, Norway.
Sterilin Ltd, Hounslow, Middlesex, UK.
Surgikos, Livingston, UK.
Unipath Ltd., Bedford, UK.
Whatman, Maidstone, Kent, UK.

2. CHEMICAL ABBREVIATIONS

ATP	Adenosine-5'-triphosphate
BCIG	5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (XGal)
BSA	Bovine serum albumin
$CaCl_2$	Calsium chloride
CO_2	Carbon dioxide
CTP	Cytidine-5'-triphosphate
CsCl	Caesium chloride
DAB	3,3'-Diaminobenzidine
ddH_2O	Double distilled water
DEPC	Diethyl-pyrocarbonate
DMP	Dimethyl formamide
DMSO	Dimethylsulphoxide
dNTP	deoxy-nucleotide-triphosphate
DTT	Dithiothreitol
EDTA	Ethylene-diamine-tetra-acetic acid
EtBr	Ethidium bromide
FITC	Fluorescein isothiocyanate
G6PD	Glucose-6-phosphate dehydrogenase
GTP	Guanosine-5'-triphosphate
HCl	Hydrochloric acid
H_2O_2	Hydrogen peroxide
I	Iodine
IPTG	Isopropyl-beta-D-thio-galactopyranoside
KCl	Potassium chloride
KH_2PO_4	Potassium di-hydrogen orthophosphate
KOH	Potassium hydroxide
$MgCl_2$	Magnesium chloride
$MgSO_4$	Magnesium sulphate
$MnCl_2$	Manganese chloride

MOPS	3-[N-Morpholino]-propane-sulfonic acid
NaCl	Sodium chloride
NaH_2PO_4	Sodium di-hydrogen orthophosphate
Na_2HPO_4	di-Sodium hydrogen orthophosphate
NaOH	Sodium hydroxide
NP40	Nonidet P40
PEG	Polyethylene glycol
PMSF	Phenyl methyl sulphonyl fluoride
$RbCl_2$	Rubidinium chloride
SDS	Sodium dodecyl sulphate
TPA	12-0-tetradecanoylphorbol-13-acetate
Tris	Tris(hydroxymethyl)aminomethane
TRITC	Tetramethylrhodamine isothiocyanate
TTP	Thymidine-5'-triphosphate
$ZnSO_4$	Zinc sulphate

3. GENERAL SOLUTIONS

All are made up in double distilled water (ddH_2O) unless stated

Phosphate buffered saline (PBS): (for immunoblotting)	0.8% (w/v) NaCl 0.02% (w/v) KCl 0.02% (w/v) Na_2HPO_4 0.15% (w/v) KH_2PO_4
PBS (for all other purposes):	made up from tablets as in manufacturers instructions (Oxoid)
Complement fixation buffer (CFB):	made up from tablets as in manufacturers instructions (Oxoid)
Tris/EDTA (TE):	10mM Tris 1mM EDTA adjusted to pH 8.0 with HCl
Tris/borate/EDTA (TBE) (10x):	0.89M Tris 0.89M Boric acid 0.02M EDTA

Standard saline citrate (SSC)(20x):	3M NaCl 0.3M tri-Sodium citrate
Standard saline phosphate EDTA: (SSPE)(20x)	3M NaCl 0.18M NaH_2PO_4 0.025M EDTA adjust pH to 7.4 with NaOH
Tris-buffered saline (TBS):	150mM NaCl 50mM Tris adjust pH to 8.0 with HCl

Phenol: buffered twice with 500mM Tris-HCl (pH 8.0), 50mM EDTA and twice with distilled water. Stored in the dark at 4°C.

Phenol/chloroform: 49.5% (v/v) buffered phenol
49.5% (v/v) chloroform
1% (v/v) iso-amyl alcohol
0.1% (w/v) 8-hydroxyquinoline
Stored in the dark at 4°C.

Formamide: deionised by mixing with ion exchange resin (Amberlite MB1, BDH) for 30 minutes and filtering. Stored at -20°C.

Tris buffers: made up as 1M stock solutions, adjusted to required pH using HCl.

4. REAGENTS

Reagents, other than those listed below, were supplied by BDH.

Reagent	Supplier
Acrylamide	Bio-Rad Laboratories
Agar	Difco Laboratories
Agarose	ICN Biomedicals
Ammonium persulphate	Bio-Rad Laboratories

Ampicillin	Sigma
ATP	Boehringer Mannheim
Bis-acrylamide	Bio-Rad Laboratories
Bromophenol blue	Sigma
BSA	Sigma
Crystal violet	Sigma
DAB	Sigma
DNA polymerase (Klenow)	Amersham International
DNase I	Boehringer Mannheim
dNTPs	Boehringer Mannheim
Ficoll-Paque	Pharmacia LKB Biotechnology
Formamide	Fluka Chemie AG
Gelatin	Sigma
IPTG	Boehringer Mannheim
Lysozyme	Sigma
Nitrocellulose	Schleicher and Schuell
NTPs	Boehringer Mannheim
Powdered skimmed milk	Marvel, Cadbury
Preservative free heparin	Painse and Byrne
Proteinase K	Boehringer Mannheim
Restriction enzymes	New England Biolabs, Boehringer Mannheim
RNase A	Boehringer Mannheim
Sephadex G50	Pharmacia LKB Biotechnology
Sephadex G75	Pharmacia LKB Biotechnology
TEMED	Bio-Rad Laboratories
T4 DNA ligase	New England BioLabs
Trypan blue	Sigma
Tryptone	Difco Laboratories
Yeast extract	Difco Laboratories

5. GENERAL PLASTICWARE

Disposable plastic pipettes	Falcon
Microfuge tubes (0.5 and 1.5ml)	Scotlab
Petri dishes	Sterilin
Plastic univeral tubes (25ml)	Sterilin
Plastic bijoux (5ml)	Sterilin
Polypropylene centrifuge tubes (50ml)	Falcon

6. EQUIPMENT

Equipment	Model and supplier
Beta-particle counter	'1216 Rackbeta', Pharmacia LKB Biotechnology
Bench top centrifuge	'CR4.11', Jouan
Cell Harvester	'Semi-automatic cell harvester', Skatron AS
CO_2 incubator	'Type 22', LEEC
Cytospin	Shandon Scientific
Electrophoresis power pack	'2197', Pharmacia LKB Biotechnology
Electroporator	'Gene Pulser', Bio-Rad Laboratories
Large capacity centrifuge	'RC-5B Superspeed', DuPont
Microfuge	'Micro Centaur', MSE
Sonicator	'Soniprep 150', MSE
Spectrophotometer	'M302', Camlab
Ultracentrifuge	'L8-55M', Beckman Instruments
Water baths	Grant Instruments
X-ray film	'Hyperfilm MP', Amersham International

7. CELL CULTURE

All cell culture reagents supplied by Flow Laboratories unless stated otherwise.

7.1 Growth medium

1x RPMI 1640 containing 2mM L-glutamine, 100IU penicillin, 100µg/ml streptomycin and 10% (v/v) mycoplasma-free heat inactivated foetal calf serum (FCS) (Biological Industries or Gibco BRL).

7.2 Wash medium

As for growth medium except for 2% (v/v) FCS.

7.3 Freezing medium

1x RPMI 1640 containing 50% FCS and 15% DMSO (BDH).

7.4 Hygromycin B

Hygromycin B (Calbiochem) was reconstituted in ddH_2O, sterilised by filtration through a 45µm filter unit (Flow Laboratories), aliquoted and stored as a stock

solution (100mg/ml) at -20°C.

7.5 Plasticware

Item	Supplier
15mm, 24 well cell culture plates	Falcon
35mm, 6 well cell culture plates	Falcon
96 well microculture plates	Falcon
Polypropylene centrifuge tubes (50ml)	Falcon
Cell culture flasks (50, 250 and 500ml)	Falcon
Plastic universal tubes (25ml)	Sterilin
Liquid nitrgen storage vials	Nunc

7.6 Cell lines

Cell lines used in these experiments are detailed in Table 14

8. ANTIBODIES

Polyclonal and monoclonal antibodies used in this study are detailed in tables 15 and 16

Table 14

Cell lines used in this study

Cell line	Type	Reference/Source
IB4	LCL	King *et al.*, (1980)
CHEP[1]	LCL	Rooney *et al.*, (1985)
ELI[1]	LCL	Rooney *et al.*, (1985)
Raji	EBV-positive BL	Pulvertaft, (1965)
Ramos	EBV-negative BL	Klein *et al*, (1975)
P3HR-1	EBV-positive BL	Hinuma *et al*, (1967)
M232[2]	LCL	N. Wedderburn
M242[2]	LCL	N. Wedderburn
M245[2]	LCL	N. Wedderburn
M287[2]	LCL	N. Wedderburn
B95-8	LCL	Miller, *et al.*, (1972)
Namalwa	EBV-positive BL	Klein and Dombos, 1973
PS	LCL	D.H. Crawford

1 CHEP and ELI LCLs were a gift from Prof. A. Rickinson (University of Birmingham).

2 M232, M242, M245 and M287 are EBV-immortalised common marmoset LCLs (gifts from Dr. N. Wedderburn, Royal College of Surgeons).

Table 15

Polyclonal antisera

Name	Specificity	Reference/source
JAT	VCA, EBNA-1	Thomas *et al.*, 1990b
WDA	EBV negative	Thomas *et al.*, 1990b
Bull	VCA, EA	D.H. Crawford[1]
RT	EBNAs 1, 2, 3, 4, 6	Allday *et al.*, 1988
115	SV40 LT	D. Lane[2]

Polyclonal sera are human except for 115 which is raised in rabbit.

1) Royal Postgraduate Medical School, London.

2) 115 antibody was a gift from Dr. D. Lane, Imperial Cancer Research Fund, London.

Table 16

Monoclonal antibodies

Antibody	Antigen	Source	Reference
J5	CD10 (cALLa)	Coulter	Ritz *et al.*, 1980
38.13	CD77 (BLA)	*	Wiels *et al.*, 1981
HD37	CD19	Dako	Pezzuto *et al.*, 1986
B1	CD20	Coulter	Stashenko *et al.*, 1980
B2	CD21	Coulter	Nadler *et al.*, 1981
TO15	CD22	Dako	Nadler 1985
MHM6	CD23	*	Rowe, M., *et al.*, 1982
BerH2	CD30	Dako	Schwab *et al.*, 1982
Ki24	CDw70	*	Stein *et al.*, 1983
BerT9	CD71	Dako	Dako
W6.32	HLA I	*	Barnstable *et al.*, 1978
L243	HLA DR	*	Lampon *et al.*, 1980.
MHM23	CD18	*	Hildreth *et al.*, 1983
MHM24	CD11a	*	Hildreth *et al.*, 1983
TS1/22	CD11a	*	Sanchez-Madrid *et al.*, 1982
TS2/9	CD57	*	Sanchez-Madrid *et al.*, 1982
RR1/1	CD54	*	Rothlein *et al.*, 1986
V9	Vimentin	Dako	Osborn *et al.*, 1984
JF186	EBNA-5	*	Finke *et al.*, 1987
CS1-4	LMP	*	Rowe, M,. *et al.*, 1987a
Myc1-9E10	c-*myc*	*	Evan *et al.*, 1985

All are mouse monoclonal antibodies except for 38.13 which is a rat monoclonal antibody.

* Antibodies were kindly supplied by the following individuals: 38.13, Dr T. Tursz, Institut Gustave-Roussy, Villejuif, France; MHM6, MHM23, MHM24, Prof. A. McMichael, University of Oxford, UK; Ki24, Prof. H. Stein, Free University of Berlin, FRG; W6.32, Dr J.A. Thomas, Imperial Cancer Research Fund, London, UK; L243, Dr. R. Lechler, Royal Postgraduate Medical School, London, UK; TS1/22, TS2/9, RR1/1, Prof. T. Springer, Harvard Medical School, Boston, USA; JF186, Prof. G. Klein, Karolinska Institute, Stockholm, Sweden; CS1-4, Dr M. Rowe, Cancer Research Campaign Laboratories, University of Birmingham, UK; Myc1-9E10, Dr G. Evan, Imperial Cancer Research Fund, London, UK

CHAPTER VI

METHODS

1. CELL CULTURE

1.1 Culture of cells

Cells were cultured in a humidified 5% CO_2 incubator at 37°C and fed with normal growth medium as required, usually every 2-3 days. Where appropriate the antibiotic hygromycin B was added to select for cells transfected with plasmid constructions expressing the hygromycin B gene, *hph* (Gritz and Davies, 1983).

1.2 Storage of cells

Cells (10^7/ml in freezing medium) were stored under liquid nitrogen in 1.5ml liquid nitrogen storage vials. Before storage under liquid nitrogen the cells were cooled to -70°C at an approximate rate of 1°C per minute. Cells were recovered from liquid nitrogen by quickly thawing in a 37°C water bath and resuspending gently by adding wash medium dropwise to a final volume of 10ml. After spinning at 500 x g for 7 minutes the cells were resuspended in 15ml growth medium and added to a 50ml cell culture flask.

1.3 Counting of cells

To assess viability 10µl of a cell suspension containing 0.25% trypan blue was counted on a light microscope using a haemocytomoeter (Weber Improved Neubauer, BDH). Cells taking up the dye were dead, those excluding the dye alive. Trypan blue was made up as a 0.5% stock solution in PBS.

1.4 Hygromycin B sensitivity assays

To determine the concentration of hygromycin B required to kill non-transfected cells, cells were plated out in triplicate at 10^4/well in 96 round bottomed microculture plates and hygromycin B added at dilutions ranging from 0 - 400µg/ml. Cells were fed every three days by replacing half the medium with fresh medium containing hygromycin B. To assess cell growth in the presence of hygromycin B, 1µCi [methyl-^{3}H]thymidine (70-85Ci/mmol, Amersham International) was added to each well after 10 days and left for 4 hours before harvesting onto a glass microfibre filter (GF/C, Whatman) using a cell harvester. Incorporation of tritiated thymidine was measured by counting on a beta particle counter.

2. TRANSFECTION OF DNA INTO CELL LINES

2.1 Electroporation

Electroporation was carried out in disposable plastic electroporator cells (0.4cm electrode gap, Bio-Rad Laboratories) using a "Gene Pulser" apparatus (Bio-Rad Laboratories). 10^6 cells were suspended in 0.8ml RPMI 1640 medium containing 10μg of the relevant DNA and added to a cuvette. After 10 minutes on ice the cells were electroporated, placed on ice for a further 10 minutes and diluted to 2ml with normal growth medium. The cells were transferred to a 15mm 24 well culture plate and cultured for 48 hours before selection by addition of hygromycin B to the relevant concentration (section 1.4). Any resistant cells were cultured and expanded. Transfected cell lines were maintained under selection to avoid loss of plasmid DNA and outgrowth of non-resistant cells.

2.2 Liposome mediated transfection

Liposome mediated transfection (Felgner, *et al*, 1987) was carried out using a commercially available reagent (Lipofectin, Gibco BRL). The method used was essentially as recommended by the manufacturers. To adhere cells to plastic, 35mm tissue culture dishes were coated with 4.5μl of a 10mg/ml solution of "Cell-Tak" (BioPolymers Inc.). After drying the dishes were immersed in 95% ethanol for 5 minutes, rinsed twice with sterile distilled water and air-dried. 4 x 10^6 cells were washed twice in serum-free "Opti-MEM I" medium (Gibco BRL), resuspended in 2ml of the same medium, and added to the "Cell-Tak" coated plate. After a 30 minute incubation at 37°C the medium, and any non-adhering cells were removed and the DNA-liposome mixture added as described below. 1μg of plasmid DNA was added to 0.5ml "Opti-MEM" I medium and mixed with 10μg of "Lipofectin" diluted in 0.5ml Opti-MEM I medium. The mixture was added to the Cell-Tak coated plate and incubated at 37°C for 5 hours. After transfection the cells were removed from the plastic by incubation with 0.05% trypsin, 0.02% EDTA (Flow Laboratories), washed, and resuspended to 10ml in normal growth medium. After 48 hours, hygromycin B, at the relevant concentration, was added and thereafter the cells fed as required.

3. BACTERIAL METHODS

3.1 Bacterial media

L-broth: 1% (w/v) tryptone
0.5% (w/v) yeast extract
1% (w/v) NaCl
adjust pH to 7.2 with NaOH, autoclave to sterilise.

PSI-broth: 2% (w/v) tryptone
0.5% (w/v) yeast extract
0.4% (w/v) $MgSO_4$
10mM KCl
adjust pH to 7.6 with KOH, autoclave to sterilise.

3.2 Culture of bacteria

Bacteria were routinely cultured on L-broth plates [L-broth containing 1% (w/v) agar and ampicillin (100μg/ml)] or in suspension in L-broth containing ampicillin (100μg/ml). Suspension cultures were grown at 37°C with vigorous shaking.

3.3 Preparation and storage of competent bacteria (Hanahan, 1985)

A single colony of DH5 bacteria (Hanahan, 1985) was picked into 5ml PSI broth and cultured at 37°C with vigorous shaking for 2-3 hours. This culture was added to 100ml of prewarmed PSI broth and shaken for a further 2-3 hours at 37°C. The cells were cooled on ice for 10 minutes, transferred to sterile 50ml polypropylene tubes and centrifuged at 2500 x g for 5 minutes at 4°C. The cells were resuspended in 30ml 100mM $RbCl_2$, 50mM $MnCl_2$, 30mM potassium acetate, 10mM $CaCl_2$, 15% (v/v) glycerol (final pH 5.8) and kept on ice for 20 minutes. The cells were respun as above and resuspended in 4ml ice-cold 10mM MOPS (pH 7.0), 10mM $RbCl_2$, 75mM $CaCl_2$, 15% (v/v) glycerol (final pH 6.8). The cells were dispensed in 200μl aliquots, snap frozen in liquid nitrogen, and stored at -70°C.

3.4 Transfection of plasmid constructions into competent bacteria (Hanahan, 1985)

200μl of previously prepared competent DH5 bacteria were thawed at room temperature until just liquid and placed on ice. After 5 minutes 100ng of plasmid DNA in 50μl TE was added to the bacteria and incubated for 20

minutes on ice. The bacteria/DNA mix was warmed to 37°C for 3 minutes followed by incubation on ice for a further 2 minutes. 0.8ml of PSI broth was added and the transfection mix incubated for 60 minutes at 37°C with gentle shaking. 100µl of the PSI broth culture was plated out onto an L-broth agar plate containing ampicillin (100µg/ml), and the plate incubated, inverted, overnight at 37°C. The following day single colonies were picked into 25ml plastic universal tubes, containing 5ml of L-broth plus ampicillin (100µg/ml), and incubated with vigorous shaking overnight at 37°C. 4ml of this culture was used to prepare plasmid DNA (section 3.5) to check the identity and integrity of the transfected DNA. The remaining 1ml of culture was diluted with 1ml of fresh L-broth, incubated for 1 hour at 37°C and used to make a bacterial stock. Stocks were prepared by diluting 1:1 with sterile glycerol and storing in 2ml liquid nitrogen storage vials at -70°C.

3.5 Small scale plasmid DNA preparations (adapted from Birnboim, 1983)

Bacteria were cultured overnight in 25ml plastic universal tubes, containing 5ml L-broth, as described earlier (section 3.2). The culture was centrifuged at 4000 x g for 5 minutes and the cell pellet resuspended in 150µg of 25mM Tris (pH8.0), 10mM EDTA (pH8.0), 1% glucose, transferred to a 1.5ml microfuge tube and left for 5 minutes at room temperature. 300µl of 200mM NaOH, 1% SDS was added and incubated for 5 minutes on ice. 225µl of 3M Potassium acetate was added and, following a 15 minute incubation on ice, the mixture was centrifuged for 5 minutes at 13400 x g in a microfuge. The supernatant was transferred to a fresh 1.5ml tube and 500µl of buffered phenol added. The tube was vortexed and centrifuged at 13400 x g for 5 minutes. The aqueous phase was transferred to a clean microtube and the plasmid DNA precipitated with ethanol (section 6.1). Following precipitation the DNA pellet was resuspended in 10µg of TE buffer. Restriction enzyme digestion of the DNA (section 6.4) was carried out to confirm the identity of the plasmid DNA.

3.6 Large scale plasmid DNA preparations (adapted from Birnboim and Doly, 1979)

One litre cultures of bacteria were centrifuged at 7000g for 10 minutes at 4°C and the cell pellet resuspended in 40ml 25mM Tris (pH8.0), 10mM EDTA (pH8.0), 50mM glucose, 5mg/ml lysozyme. After 5 minutes at room temperature 80ml of 200mM NaOH, 1% SDS was added and incubated for a further 5 minutes on ice. 40ml of ice cold 3M potassium acetate was added, incubated for

15 minutes on ice and then centrifuged at 7000 x g for 15 minutes at 4°C. The supernatant was decanted through gauze, mixed with 96ml of propan-2-ol and centrifuged at 7000 x g for a further 15 minutes at 4°C. The supernatant was discarded and the pellet dried and resuspended in 10ml TE buffer. Following two phenol/chloroform extractions (section 6.2) the aqueous phase was precipitated with ethanol (section 6.1). Following precipitation the pellet was resuspended in 10ml TE buffer and 10g CsCl plus 1ml EtBr (5mg/ml) added. This was added to a 16x76mm sealable polyallomer tube ('Quick-Seal', Beckman Instruments) The tube was heat sealed and centrifuged at 220000 x g for 48 hours at 20°C in an ultracentrifuge, using a 70Ti rotor (Beckman Instruments). Following ultracentrifugation the plasmid DNA was visible as two discrete bands. The lower band, representing the supercoiled form, was collected using a 5ml hypodermic syringe. EtBr was removed by repeatedly mixing with CsCl saturated propan-2-ol and discarding the upper layer until the lower aqueous layer was clear of all EtBr. The aqueous phase was dialysed with TE buffer for 4 hours at 4°C with hourly changes of TE buffer. The resulting dialysate was precipitated with ethanol and the pellet resuspended in 0.5ml sterile TE buffer. The amount of DNA present was quantified by spectrophotomoetry (section 6.3) and the concentration adjusted to 1mg/ml. Restriction enzyme analysis of 1µg of the DNA was performed to confirm the identity of the plasmid DNA (section 6.4). DNA was stored in sterile microfuge tubes at -20°C.

4. EXTRACTION AND ANALYSIS OF RNA

4.1 Preparation of RNAse free solutions and equipment

To avoid degradation of RNA by contaminating RNases, several precautions were taken (Blumberg, 1982). Wherever possible, sterile disposable plastic pippettes and bottles were used. All glassware used was treated by soaking in 0.1% DEPC overnight, and baked at 250°C for 3 hours. All solutions were made up using water which had been treated overnight with DEPC (0.1% v/v) and autoclaved for 15 minutes. Electrophoresis tanks were soaked in H_2O_2 (5%) for 30 minutes, and rinsed with DEPC-treated ddH_2O. For all proceedures disposable gloves (Microtouch, Surgikos) were worn.

4.2 RNA extraction (Maniatis, *et al.*, 1982)

Cells were fed with fresh growth medium 24 hours prior to harvesting, to ensure cells were in log phase growth. Cells were harvested, washed twice with PBS, and the cell pellet resuspended in 5 volumes of 4M guanidinium

isothiocyanate, 5mM sodium citrate (pH 7.0), 0.1M beta-mercaptoethanol, 0.5% (v/v) sarkosyl. The cells were homogenised using a dounce homogeniser (BDH) and 1g CsCl added per 2.5ml homogenate. The homogenate was gently layered over a 1.2ml cushion of 5.7M CsCl, 0.1M EDTA (pH 7.5) in a 13 x 51mm polyallomer ultracentrifuge tube (Beckman Instruments), and centrifuged in an SW55Ti rotor (Beckman Instruments) at 170000 x g for 16 hours at 20°C. Following centrifugation the supernatant was discarded, the tube dried, and the RNA pellet resuspended in 5ml Tris/EDTA/SDS [10mM Tris (pH 7.4), 5mM EDTA (pH 7.5), 1% SDS]. This was extracted once with chloroform/butanol (4:1) and the upper aqueous phase transferred to a clean 30ml glass tube (Corex, DuPont). The organic, lower phase was re-extracted with 5ml of Tris/EDTA/SDS and the two aqueous phases combined and precipitated with ethanol at -20°C overnight. The RNA was pelleted by centrifugation at 16500 x g and resuspended in 0.5ml TE buffer. The RNA was quantified by spectrophotometry (section 6.3), and stored at -70°C.

4.3 Purification of mRNA

To enrich for mRNA in the total RNA extracted from cells (section 4.2) a commercial kit was used ('mRNA Purification Kit', Pharmacia LKB Biotechnology). The proceedure followed was exactly as described in the manufacturers instructions, with the exception that only one round of purification was used, which results in a 10 fold enrichment of poly-adenylated RNA.

4.4 Electrophoresis of RNA

RNA was separated by gel electrophoresis in gels containing 1% agarose, 20mM MOPS (pH 7.0), 1mM EDTA (pH 7.5), 0.74% formaldehyde, 5mM iodoacetamide and 100µg EtBr (Dr J. Allen, Royal Postgraduate Medical School, London, personal communication). 10µg of poly-adenylated enriched RNA (section 4.3) in 40µl sample buffer [20mM MOPS (pH 7.0), 1mM EDTA, 3.7% (v/v) formaldehyde, 10% (v/v) deionised formamide, 1.5% (w/v) bromophenol blue] was heated to 80°C for 5 minutes and loaded into a preformed gel slot. Electrophoresis was in 20mM MOPS (pH 7.0), 1mM EDTA at a constant current of 80mA. When the bromophenol blue dye front reached the end of the gel the gel was removed, immersed in 100mM NaOH for 30 minutes, and neutralised for a further 30 minutes in 0.2M glacial acetic acid. At this stage the 18S and 28S ribosomal RNA species could be visualised under U/V light and photographed to provide size markers.

4.5 Transfer of RNA to nylon membrane

Following electrophoresis RNA was transferred to a nylon membrane ('Zeta-probe', Bio-Rad Laboratories). The gel was laid on top of a double layer of 3MM (Whatman) paper, the ends of which were dipped into a reservoir of 20x SSC. The nylon membrane, pre-wetted in ddH_2O and 20x SSC, was placed on top of the gel, and covered with 2 sheets of dry 3MM paper and a 10cm deep layer of paper towels. These were weighted down with a 500g weight and left overnight. The following day the membrane was rinsed briefly in 5x SSC, air-dried between 2 sheets of 3MM paper and heated for 2 hours at 80°C.

4.6 Labelling of DNA probes with radio-labelled nucleotide

Double stranded DNA probes were labelled with [^{32}P]dCTP by random oligonucleotide priming (Feinberg and Vogelstein, 1983) using a commercially available kit ("Multiprime DNA labelling system", Amersham International). 25ng of DNA in 10µl of TE was heated for 3 minutes at 100°C and cooled on ice. The DNA was added to a reaction mix containing dATP, dGTP, dTTP, random hexanucleotide primers, BSA, [^{32}P]dCTP (Amersham International) and DNA polymerase I ('Klenow' fragment), exactly as described in the manufacturers' instructions. After a 30 minute incubation at 37°C the labelled DNA was separated from unincorporated nucleotides by passing the reaction mix through a disposable plastic column (Bio-Rad Laboratories) containing sephadex G-75 beads (Pharmacia LKB Biotechnology) pre-equilibrated with 150mM NaCl, 10mM EDTA (pH 8.0), 50mM Tris (pH 7.5), 0.1% (v/v) SDS. The fractions containing the labelled probe were pooled and denatured by heating to 100°C for 3 minutes, followed by cooling on ice. The labelled probe was added immediately to the hybridisation mix (section 4.7)

4.7 Hybridization of DNA probe to immobilized RNA

RNA immobilized to nylon membranes was pre-hybridized for 3 hours at 42°C in 5x SSPE, 50% deionised formamide, 0.1% (w/v) skimmed milk powder, 0.2% (v/v) SDS (Dr S. Legon, Royal Postgraduate Medical School, London, personal communication). After 3 hours the pre-hybridisation solution was removed and replaced with fresh pre-hybridization solution containing radio-labelled probe (section 4.6). Following a 72 hour incubation at 42°C the hybridization mix was discarded and the membrane washed twice in 0.2x SSC, 0.2% SDS for 30 minutes at room temperature, followed by two further 30 minutes wash in 0.2x SSC, 0.2% SDS at 68°C. After washing the membrane was wrapped in a clear plastic film ("Saran-Wrap", Dow Chemical Co.), without allowing the membrane to dry, and exposed to pre-flashed X-ray film.

4.8 Removal of hybridised probe

After exposure the bound probe was removed by incubating in TE buffer containing 0.1% SDS at 95°C for 30 minutes. The membrane was then re-hybridised with different probes as described above.

5. DNA PROBES

5.1 LFA-1 alpha

To probe for LFA-1 alpha subunit mRNA, a 1.8kb EcoR1 fragment (3R1) from the 5' end of an LFA-1 alpha cDNA clone was used (Larson *et al*, 1989) (3R1 was a gift from Prof. T. Springer, Harvard University, Boston, USA).

5.2 LFA-1 beta

A 700bp BamH1-EcoR1 fragment (J8) excised from a 2.7kb cDNA clone (J-9) was used to probe for LFA-1 beta subunit mRNA. (Law *et al.*, 1988) (a gift from Dr A. Law, University of Oxford).

5.3 C-*myc*

In northern blot experiments a plasmid DNA construction (pSV-Hu-c-myc-1) was used to probe for c-*myc* transcripts (Land *et al.*, 1986). pSV-Hu-c-myc-1 was a gift from Dr D. King (Royal Postgraduate Medical School, London). A single stranded M13 DNA probe containing a 414bp PstI-PstI fragment of DNA, complementary to c-*myc* exon II sense transcripts, was used in nuclear run-on experiments (Bentley and Groudine, 1986a) (gift from Dr D. Bentley, Imperial Cancer Research Fund). To probe for c-*myc* exon I sequences amplified from cDNA by polymerase chain reaction pHEBoSVmyc1,2,3 (Lombardi *et al.*, 1987) was used (chapter II, section 1.2.4).

5.4 G6PD

As a control probe a 200bp BamH1-PstI fragment isolated from a G6PD cDNA was used (Persico *et al.*, 1986) (a gift from Dr. P. Mason, Royal Postgraduate Medical School, London).

6. GENERAL NUCLEIC ACID METHODS

6.1 Ethanol precipitation of nucleic acids (Maniatis *et al.*, 1982)

DNA or RNA, dissolved in TE, was precipitated by adding 0.1 volumes of 3M sodium actetate and 2.2 volumes of ice-cold 95% ethanol, mixing well and leaving for 2 hours at -70°C or overnight at -20°C. Small scale precipitations (<1.5ml) were centrifuged for 10 minutes in a microfuge (13400 x g) to pellet the nucleic acid. Larger scale precipitations (1.5-30ml) were carried out in glass centrifuge tubes (Corex, Dupont) in a large capactiy centrifuge (16500 x g, 30 minutes, 4°C). The supernatant was discarded and the pelleted nucleic acid washed with 70% ethanol, re-centrifuged, the supernatant discarded and the pellet left to dry. The DNA or RNA was resuspended in TE, its concentration determined by spectrophotometry (section 6.3), and resuspended to the required concentration in TE.

6.2 Phenol/chloroform extraction

Protein was removed from aqueous solutions of nucleic acid by extraction with buffered phenol/chloroform. The nucleic acid solution was mixed with an equal volume of buffered phenol/chloroform and centrifuged at 1600 x g for 15 minutes at room temperature. Following centrifugation the upper, aqueous phase containing the nucleic acid was retained taking care not to disturb the interphase.

6.3 Quantitation of nucleic acids (Maniatis *et al.*, 1982)

Quantitation of DNA and RNA was performed by spectrophotometry, determining optical densities at 260 and 280nm. An OD_{260} of 1 is equivalent to 50µg/ml for double stranded DNA and 40µg/ml for RNA or single stranded DNA. The OD_{260}/OD_{280} ratio is used to indicate purity of sample. A pure sample of DNA should give a ratio of 1.8 and RNA a ratio of 2.0.

6.4 Restriction endonuclease digestions

Digestion of double stranded DNA with restriction enzymes was carried out in autoclaved 1.5ml microtubes as recommended by the suppliers. 1µg DNA was digested in 10µl TE containing 3 units of restriction enyme, NaCl (0, 50 or 100mM depending on enzyme used), 10mM $MgCl_2$, 1mM DTT, 10mM Tris pH 7.5 (50mM if 100mM NaCl used). Reactions were incubated at 37°C for 90

minutes, and digestion halted by addition of 0.1 volumes of DNA loading buffer [1.25% (w/v) SDS, 2.5mM EDTA, 37.5% (w/v) sucrose, 0.15% (w/v) bromophenol blue]. For larger amounts of DNA reaction volumes were scaled up accordingly. If not analysed immediately by agarose gel electrophoresis, samples were stored at -20°C.

6.5 Agarose gel electrophoresis of DNA

DNA, digested with restriction endonucleases, was separated in agarose gels using 'minigels'. Gels (10 x 8cm) were made up by dissolving the relevant amount of agarose (dependent on size of DNA fragments to be separated, Maniatis *et al.*, 1982) in 50ml 1x TBE, by heating in a microwave oven. After cooling to 50°C, 25µg EtBr was added and the gel mix poured into a minigel apparatus (Flowgen Instruments). Wells for loading samples were created using a perspex 'comb'. After setting, the gel was submerged in 1x TBE containing 0.5µg/ml EtBr. The DNA sample (section 6.4) was added to the preformed wells and the samples separated using a constant current of 60mA. 0.5µg of HinDIII digested lamda phage DNA, or HaeIII digested phiX174 DNA, were used as molecular weight markers (New England BioLabs). The relative positions of the DNA fragments were visualised on a long wave U/V trans-illuminator (Ultra-Violet Products Inc.), and a permanent photographic record taken if required, using Polaroid Land 57 film.

6.6 Trough elution of DNA fragments

To recover restriction endonuclease digested fragments from agarose gels, the trough elution method was used (Ogden and Adams, 1982). After digestion with restriction endonucleases, the DNA was separated on an agarose gel and visualised on a U/V transilluminator. A slot (2mm deep) was cut immediately in front of the required band using a sterile, disposable scalpel, and the slot filled with 2x TBE. The current was reapplied and the band of DNA allowed to run on into the slot. The 2x TBE containing the DNA was removed from the slot and transferred to a 1.5ml microfuge tube. Agarose fragments were removed by centrifugation in a microfuge (3350 x g) and the supernatant containing the DNA extracted with phenol/chloroform, precipitated with ethanol, and resuspended in TE buffer. The DNA recovered was of sufficient quality for use as a probe, or for ligation reactions.

6.7 Ligation reactions

Ligation of isolated DNA fragments (section 6.6) into vector DNA digested with the relevant restriction enzyme(s) (section 6.4) was carried out with 0.3µg insert and 0.1µg vector in 20µl ddH_2O containing 50mM Tris (pH 7.4), 10mM $MgCl_2$, 10mM DTT, 1mM ATP and 400 units T_4 DNA ligase. Reactions were incubated overnight at 16°C prior to transfection into competent bacteria (section 3.4).

7. M13 METHODS

7.1 Solutions

2x TY:	1.6% tryptone 1.0% yeast extract 0.5% NaCl in ddH_2O
H-Top agar:	0.8% agar 1.0% tryptone 0.8% NaCl in ddH_2O
H plates:	1.5% agar 1.0% tryptone 0.8% NaCl in ddH_2O
10x TM:	100mM Tris (pH 8) 100mM $MgCl_2$ in ddH_2O, stored at -20°C
10x ligation buffer:	500mM Tris (pH 7.5) 100mM $MgCl_2$ 10mM DTT in ddH_2O, stored at -20°C

7.2 Primer extension of M13

M13 template DNA was made double stranded by primer extension. M13 primer (2µg, Amersham International) and 1µl 10x TM buffer were added to 7µl

ddH_2O containing 1µg M13 template. The reaction was left for 30 minutes at 60°C to allow the primer to anneal, cooled for 5 minutes at room temperature and 0.5µl 10x TM buffer, 1µl DNA polymerase I 'Klenow' fragment and 2.5mM of each of dCTP, dGTP, dATP, dTTP added. Following a 20 minute incubation at 37°C, the reaction mix was incubated at 68°C for 20 minutes to inactivate the DNA polymerase.

7.3 Digestion of M13 DNA

To 10µl double stranded M13 DNA (section 7.1) 0.5µl 10x TM buffer, 100mM NaCl, 0.75mM DTT and 12 units of the relevant restriction endonucleases were added. This was incubated for 20 minutes at 37°C and 20 minutes at 68°C. 30µl ddH_2O was added to bring the final reaction volume up to 50µl. An equal volume of freshly buffered phenol was added, vortexed for 30 seconds and centrifuged at 13400 x g in a microfuge. The upper, aqueous, phase was transferred to a fresh 1.5ml microtube, the DNA precipitated with ethanol, and the precipitate resuspended in 50µl TE buffer.

7.4 Ligation of M13 DNA

Restriction endonuclease-digested, double stranded M13 DNA was religated by mixing 1µl M13 DNA (1mg/ml), 2µl ligation buffer, 2µl 10mM ATP, 0.5µl T4 DNA ligase and 14.5µl distilled water. As a control an identical reaction mix without T4 DNA ligase was made up. Reaction mixes were incubated overnight at 16°C, and stored at -20°C until required for transfection into competent JM101 bacteria (section 7.5).

7.5 Preparation of competent JM101 bacteria

JM101 bacteria (Yanisch-Perron, *et al.*, 1985) were cultured in 2x TY medium standing overnight at 37°C. 1ml of the overnight culture was diluted 1:50 with 2 x TY and incubated shaking at 37°C for a further 3 hours. The cells were centrifuged for 5 minutes at 2700 x g and the supernatant discarded. The pellet was resuspended in 25ml 50mM $CaCl_2$ and left on ice for 20 minutes. After a further 5 minute spin at 2700 x g, the cells were resuspended in 5ml 50mM $CaCl_2$ ready for transfection.

7.6 Transfection of JM101

10µl of ligation reactions (section 7.4) were added to 300µl aliquots of competent JM101 cells and left on ice for 40 minutes. Following incubation at 42°C for 90 seconds, the transfected cells were added to 3ml top agar containing 30µl 2% BCIG (X-Gal) in DMF, 30µl IPTG and 200µl freshly cultured JM101 cells. The

mix was poured onto H-plates, allowed to set and incubated, inverted, at 37°C overnight. M13 phage containing inserted DNA were visible as white plaques.

7.7 Culture of phage and purification of template DNA

White plaques were picked into sterile plastic bijou tubes containing 2ml of a 1:100 dilution of an overnight culture of competent JM101 cells, and grown at 37°C for 5 hours with vigorous shaking. The cultures were transferred to sterile 1.5ml microtubes, centrifuged at 13400 x g for 5 minutes and the virus-containing supernatant transferred to a fresh tube. To this, 200µl of 20% polyethylene glycol (PEG) (Sigma) in 2.5M NaCl was added, mixed well and incubated on ice for 15 minutes. After spinning for a further 10 minutes at 13400 x g the supernatant was discarded and the pellet resuspended in 100µl of TE buffer. This was extracted with an equal volume of freshly buffered phenol and the aqueous phase precipitated with ethanol. After precipitation the pellet was resuspended in 30µl of TE buffer and stored at -20°C, prior to sequencing to check the identity of the M13 clone, and use as a template for generating a labelled probe for S1 endonuclease mapping of RNA transcripts (section 8.2).

8. S1 ENDONUCLEASE MAPPING OF RNA TRANSCRIPTS (Davis *et al.*, 1986)

8.1 Solutions

Hybridisation buffer:	20mM Tris (pH 7.4) 400mM NaCl 1mM EDTA in ddH_2O
5x S1 buffer:	1.5M NaCl 16.6mM ZnSO4 300mM sodium acetate (pH 4.5) in ddH_2O

Formamide loading buffer:	10ml deionised formamide
	0.01g xylene cyanol
	0.01g bromophenol blue
	0.5ml 0.2M EDTA

8.2 Preparation of probe and markers

Double-stranded DNA was prepared from probe template M13 by primer extension using the method described in section 7.2, except for substitution of unlabelled dCTP with 50μCi [^{32}P]dCTP. Radio-labelled probe (M13myc.i1.e2) was obtained by addition of DTT to 1mM and digestion of the primer extension reaction with 24 units of the relevant restriction endonucleases. After incubation at 37°C for 60 minutes, the reaction mix was added to 20μl formamide loading buffer, heated to 80°C, and loaded onto a 8M urea, 5% polyacrylamide gel. The gel was electrophoresed in 1x TBE at a constant current of 27mA until the dye front was near the bottom of the gel.

The position of the labelled probe was determined by exposing a sheet of X-ray film to the gel for 5 minutes, and using the developed X-ray film as a template for excising the probe. The portion of gel containing the probe was carefully excised using a sterile disposable scalpel and transferred to a sterile microtube. The DNA was extracted by adding 750μl of 0.5M ammonium acetate, 10mM magnesium acetate, 1mM EDTA, 0.1% SDS, 7.5μg yeast tRNA (Boehringer Mannheim), and incubating overnight at 37°C. The extraction mixture was centrifuged in a microfuge for 5 minutes at 13400 x g and the supernatant transferred to a fresh microtube. Incorporation of ^{32}P, assessed by counting a small aliquot in a beta-counter, was approximately 10^6 cpm. Expected incorporation was between 3.5×10^5 and 1.5×10^6 cpm (Davis, *et al.*, 1986).

Markers were generated by digesting pBR322 DNA with MspI and labelling with ^{32}P dCTP. 1μg pBR322 DNA was digested with the restriction enzyme MspI for 30 minutes at 37°C. 1mM dGTP (Boehringer Mannheim), 1μCi [a-^{32}P]dCTP and 0.5μl DNA polymerase I ('Klenow' fragment) was added, and incubated at room temperature for 15 minutes. The reaction was halted by heating to 68°C for 10 minutes. 1μl of the reaction mix was added to 5μl formamide loading buffer and run on the gel, in parallel with the probe.

8.3 Hybridization and S1 endonuclease digestion

20μg total RNA and 100μl probe (section 8.2) were co-precipitated with ethanol and resuspended in 105μl hybridisation buffer plus 15μl ddH_2O. Following heating to 75°C for 15 minutes the probe and RNA were hybridised overnight at 55°C in 0.5ml microfuge tubes. As a control, probe was hybridised to 20μg yeast tRNA.

To each hybridisation 100 units S1 endonuclease (Boehringer Mannheim), 280μl ddH_2O, 1μg salmon sperm DNA (2mg/ml), 80μl 5 x S1 buffer was added, mixed, and incubated at 37°C for 60 minutes. As a control probe alone was mixed with S1 endonuclease and S1 digestion buffer. The digestion mixes were then extracted once with buffered phenol/chloroform and precipitated with ethanol. The precipitates were resuspended in 4μl TE buffer and 4μl formamide loading buffer and heated to 80°C for 15 minutes. The samples were loaded onto a 8M urea, 5% polyacrylamide gel, along with labelled markers (section 8.2). The gel was electrophoresed in 1x TBE at a constant current of 27mA until the dye front was 2-3 inches from the bottom of the gel.

8.3 Detection of protected transcripts

After electrophoresis the gel was immersed in 10% acetic acid for 15 minutes, transferred to a sheet of 3MM paper (Whatman), covered with 'Saran Wrap' and dried on a vacuum gel drier (Bio-Rad Laboratories) for 90 minutes at 80°C. The gel was exposed to X-ray film for 72 hours.

9. NUCLEAR RUN-ON EXPERIMENTS (Eick and Bornkamm, 1986)

9.1 Preparation of nuclei

10^7 cells were washed 3 times in ice cold PBS and resuspended in 1ml 10mM Tris (pH 7.4), 10mM NaCl, 3mM $MgCl_2$, 0.5% NP40. After incubating on ice for 5 minutes the nuclei were centrifuged at 500 x g for 5 minutes, the supernatant discarded, and re-washed in 1ml of the above buffer. The pelleted nuclei were resuspended in 100μg of 50mM Tris (pH 8.0), 40% glycerol, 5mM $MgCl_2$, 0.1mM EDTA, and stored in liquid nitrogen until required.

9.2 Transcription reactions

10^7 nuclei, prepared as in section 9.1, were thawed on ice and mixed with an equal volume of 10mM Tris (pH 8.0), 5mM $MgCl_2$, 300mM KCl, 0.5mM ATP, 0.5mM GTP, 0.5mM CTP, 100µCi [^{32}P]UTP (800Ci/mmol) (Amersham International). The reaction was incubated for 25 minutes at 28°C and DNAse I (Boehringer Mannheim) added to 10µg/ml. The reaction was incubated for a further 5 minutes at 28°C and 100mM Tris (pH 7.5), 50mM EDTA, 1% SDS and 50µg proteinase K (Boehringer Mannheim) added. After a 60 minute incubation at 37°C the labelled transcripts were separated from unincorporated nucleotides by passing the reaction through a disposable plastic column containing sephadex G50 (Pharmacia LKB Biotechnology), pre-equilibrated with 10mM Tris (pH 7.5), 1mM EDTA, 1% SDS.

9.3 Preparation of probe DNA

DNA for hybridisation to transcription reactions were immobilised on a nylon membrane ('Zeta-probe', Bio-Rad Laboratories) using a slot blot apparatus (Schleicher & Schuell). 1µg of each DNA in 200µl 1x SSC was applied to the nylon membrane by suction through the slot-blot apparatus. Prior to applying to the membrane, double stranded DNA probes were denatured by heating to 100°C for 3 minutes and cooling rapidly on ice. The nylon membrane was air-dried and baked for 2 hours at 80°C.

9.4 Hybridisation of transcription reactions to probe DNA

Immobilized probe DNA was pre-hybridised in 5ml of 5x SSPE, 50% (v/v) deionised formamide, 0.1% (w/v) dried milk powder (Marvel), 0.2% (v/v) SDS and incubated at 42°C for 3 hours. The pre-hybridisation mix was discarded and 5ml of fresh pre-hybridisation mix containing the labelled transcription reactions was added and hybridised to the membrane for 72 hours at 42°C. Prior to adding, the transcription reactions were heated to 100°C for 3 minutes and cooled rapidly on ice.

10. POLYMERASE CHAIN REACTION (PCR)

Generation of cDNAs from total cellular RNA and PCR from primers was essentially as described by Kawasaki *et al* (1990).

10.1 Generation of cDNA by reverse transcription

Three µg of total cellular RNA was added to 20µl ddH_2O containing 10mM Tris (pH 8.3), 50mM KCl, 1.5mM $MgCl_2$, 0.001% gelatin, 5mM dNTP, 2nM random hexanucleotides (Pharmacia), 20 units RNasin (Promega Biotech), and 10 units reverse transcriptase (Super RT, Anglian Biotechnology). This was incubated for 10 minutes at 25°C, 60 minutes at 42°C, 5 minutes at 94°C and 5 minutes at 25°C in a PCR machine (PTC 100, MJ Research, USA).

10.2 Synthesis of oligonucleotides

Oligonucleotides primers (20mers) were synthesised on an ABI 380B DNA synthesizer by Dr M. Jones (Royal Postgraduate Medical School, London).

10.3 Reaction conditions

Reverse transcribed cDNAs (10µl, section 10.1) were added to 40µl ddH_2O containing 10mM Tris (pH 8.3), 50mM KCl, 1.5mM $MgCl_2$, 0.001% gelatin, 1µg of each primer (section 10.2) and 2.5 units of *Taq* DNA polymerase (Cetus Corporation). Thirty cycles of 94°C (30 seconds), 60°C (30 seconds), 72°C (2 minutes) were followed by a 5 minute incubation at 72°C and 5 minutes at 25°C. Reactions were carried out in 0.5ml microfuge tubes in a PCR machine. To prevent evaporation 50µl of mineral oil (Sigma) was layered over the reaction mix. PCR products were recovered by mixing with 200µl chloroform and retaining the upper aqueous phase.

10.4 Identification of PCR products

15µl of each completed PCR reaction (section 10.3) was electrophoresed through a 1.8% agarose gel (50ml 1 x TBE buffer containing 0.9g agarose and 50µg EtBr). Electrophoresis was at 60mA (constant current) in 1x TBE. Following electrophoresis the gel was photographed on a U/V transilluminator using a Polaroid Land camera. The gel was denatured for 60 minutes in 0.5M NaOH, 0.5M NaCl and neutralised for 120 minutes in 1M Tris (pH 8.0), 0.5M NaCl. Denatured DNA was transferred to a nylon membrane (Zeta-Probe, BioRad) using the same method as for RNA (section 4.5). DNA sequences were detected using ^{32}P labelled probes as described in sections 4.6 and 4.7, except that the hybridisation mix consisted of 7% SDS, 1mM EDTA, 263mM Na_2HPO_4, 1%

BSA (Westneat *et al.*, 1988). Prehybridisation was for 3 hours at 68°C and hybridisation overnight at 68°C.

11. IMMUNOFLUORESCENCE METHODS

Details of antibodies used in these techniques are given in chapter V, section 8.

11.1 Immunoglobulin kappa and lambda light chains.

Cells were washed twice in PBS and resuspended in PBS (10^6 cells/ml). Slide preparations of 5 x 10^4 cells were made by centrifugation at 2300 x g in a cytospin. Slides were air-dried and fixed for 3 minutes in methanol at room temperature. To each slide 25µl of mouse monoclonal antibodies against immunoglobulin kappa or lambda light chain molecules (Unipath) were added. The antibodies were diluted 1:100 in PBS. After a 30 minute incubation at 37°C the slides were washed for 10 minutes in PBS, and FITC-conjugated sheep anti mouse Ig (Sigma) (diluted 1:50 in PBS) added. Following a further 30 minute incubation and 10 minute wash the slides were mounted under a coverslip with PBS/glycerol (1:1), and examined on a U/V microscope at x400 magnification.

11.2 EBV nuclear antigen (EBNA)

The EBNA complex was detected by anti-complement immunofluorescence using an adaptation of the method described by Reedman and Klein (1973). Cells were washed twice in complement fixation buffer (CFB), and resuspended in CFB. Slide preparations, containing 5 x 10^4 cells, were prepared on a cytospin as described above (section 11.1). The slides were fixed in methanol and acetone (1:1) at -20°C for 3 minutes, and stored at -20°C in an airtight box until required. Complement was prepared by allowing peripheral blood from an EBV seronegative donor (WDA) to clot at room temperature and then incubating at 4°C for 2 hours. The serum was removed, aliquoted into suitable volumes and stored at -70°C. The complement was removed from the freezer immediately prior to use, and not refrozen.

EBNA was detected by adding an EBNA positive human polyclonal serum (JAT), diluted 1:10 in CFB containing 10% human complement. The slides were incubated for 60 minutes at 37°C and washed for 20 minutes in CFB with a change of CFB after 10 minutes. An enhancement step (Klein, *et al.*, 1976) was used, in which CFB, containing 10% (v/v) complement, was added to the slide

and incubated for 20 minutes at 37°C. After washing the slides as before 25µl FITC-conjugated goat anti-human C3c (Nordic) diluted 1:20 in CFB was added and incubated for 60 minutes at room temperature. Following washing in CFB as before, the slides were mounted in PBS/glycerol (1:1) and examined on a U/V microscope at x400 magnification.

As a negative control, serum from a known EBV seronegative donor was used.

11.3 EBV viral capsid antigen (VCA)

The method used for detecting VCA and titrating serum antibodies to VCA is an adaptation of the one originally described by Henle and Henle (1966). For titrating antibody levels, cells from the P3HR-1 cell line were washed twice in PBS and resuspended to 10^6 cells/ml in PBS. 50µl aliquots were added to 12 well polytetrafluoroethylene (PTFE) coated glass slides (Hendley), air-dried and fixed in acetone for 10 minutes at room temperature. For detecting VCA in cell lines, cytospin preparations were made as descibed in section 11.1 and slides were fixed in acetone for 10 minutes at room temperature. Slides were stored in an air-tight box at -20°C until required.

Staining for the presence of VCA in cell lines was carried out using a VCA positive polyclonal human serum (JAT, diluted 1:10 in PBS). Diluted JAT serum (10µl) was added to the slide and incubated at 37°C for 60 minutes. Slides were washed for 10 minutes in PBS and incubated with 10µl FITC-conjugated goat anti-human IgG (Dako, diluted 1:50 in PBS) for 60 minutes at room temperature. Following a 10 minute wash as before, the slides were mounted in PBS/glycerol (1:1) and examined on a U/V microscope at x400 magnification. Serum from an EBV seronegative donor (WDA), diluted 1:10 was used as a negative control.

Titration of sera for levels of IgG and IgA class antibodies to VCA was carried out by making doubling dilutions of sera in PBS, ranging from 1:8 to 1:1024, and adding 10µl of the relevant dilutions to the 12 well slides. The slides were incubated at 37°C for 60 minutes, washed for 10 minutes in PBS and 10µl of FITC-conjugated goat anti-human IgG, or IgA, (Dako, diluted 1:50 in PBS) added. Following a 60 minute incubation at room temperature, the slides were washed as before, mounted in PBS/glycerol (1:1) and examined under a U/V microscope at x200 magnification. Titration end points were taken at the point where the intensity of fluorescence began to diminish (Edwards, 1982). Known

positive and negative control sera were included with every set of titrations.

IgM class antibodies to VCA were detected using a commercially available kit (Gull Laboratories), exactly as described in the instructions supplied with the kit.

11.4 EBV early antigen (EA) complex

Detection of the EA complex in cell lines, and titration of serum antibody levels, was carried out by indirect immunofluorescence using an adaptation of the method originally described by Henle W., *et al* (1970).

For titration of antibody levels, cells from the BL cell line, Raji, were treated with TPA (20ng/ml) 2-3 days before harvesting, resulting in induced expression of EA in a small proportion of cells. Raji is a cell line which does not express VCA, but does express EA when induced with TPA (Zur Hausen *et al.*, 1978). Slides were prepared exactly as for VCA and titration of sera and staining procedures were as described for VCA (section 11.3).

EA in cell lines was detected using a polyclonal human serum (Bull). As controls JAT (VCA positive, EA negative) and WDA (VCA and EA negative) were used. Staining proceedure was as described for VCA (section 11.3).

11.5 EBV latent membrane protein

Cells were washed twice in CFB and cytospin preparations made (section 11.1). Cells were fixed in methanol and acetone (1:1) at -20°C for 3 minutes. Anti-LMP mouse monoclonal antibody (CS1-4, 20µl cell culture supernatant, used neat) was added and slides left for 60 minutes at 37°C. After washing for 3 x 10 minutes in CFB 20µl TRITC goat anti-mouse Ig (Dako) (diluted 1:50 in CFB) was added and incubated for 60 minutes at room temperature. The slides were washed as before, mounted with PBS/glycerol (1:1), and examined under a U/V microscope at x1000 magnification.

11.6 Vimentin

Vimentin in cells was detected by making cytospin preparations and fixing slides as for EBNA (section 11.2). Mouse monoclonal antibody against vimentin (Dako), diluted 1:10 in PBS, was added and the slides incubated for 60 minutes at 37°C. Slides were washed for 10 minutes in PBS and TRITC-conjugated goat

anti-mouse Ig (Dako) (diluted 1:50) added and incubated for 60 minutes at room temperature. Following a further 10 minute wash in PBS the slides were mounted in PBS/glycerol (1:1) and examined under a U/V microscope.

11.7 SV40 large T antigen.

Cells were washed twice in PBS, and slide preparations made by centrifuging at 2300 x g for 5 minutes on a cytospin. After air-drying the slides were fixed for 3 minutes in acetone and methanol (1:1) at room temperature. The cell preparations were pre-incubated for 30 minutes in PBS/gelatin/BSA [PBS containing 0.25% (w/v) gelatin and 0.25% (w/v) BSA] before being incubated for 30 minutes with a rabbit anti-SV40 large T antibody (115) diluted 1:50 in PBS/gelatin/BSA. The slides were washed for 30 minutes in PBS followed by a 10 minute wash in PBS/gelatin/BSA and incubated for a further 30 minutes at room temperature with FITC-conjugated goat anti-rabbit antibody (Dako, diluted 1:50 in PBS). After washing for 30 minutes in PBS the slides were mounted in PBS/glycerol (1:1) with a coverslip and examined on an U/V microscope at x400 magnification.

12. FLOW CYTOMETRIC ANALYSIS

12.1 Staining of cells

All washing and incubation steps were carried out in a wash solution of PBS containing 1% BSA and 0.05% sodium azide, at 4°C. Staining was carried out in 96 round-bottomed microtitre plates (Falcon).

5 x 10^5 cells were washed twice and resuspended in 50µl wash solution containing the relevant mouse monoclonal antibody, at saturating concentration, diluted in wash solution. The dilution of antibody used was determined by titration. Cells were incubated for 30 minutes at 4°C, washed 3 times and resuspended in 50µl FITC conjugated sheep anti-mouse IgG (Sigma), diluted 1:100 in wash solution. Following a further 30 minute incubation period at 4°C, the cells were washed twice and resuspended in 50µl PBS containing 2% (v/v) foetal calf serum and 2% (w/v) paraformaldehyde. After 5 minutes at 4°C the cells were rewashed and resuspended in 500µl wash solution, ready for flow cytometric analysis. If not analysed immediately cells were stored in the dark at 4°C for up to 3 days with no appreciable decrease in staining intensity. As a negative control each cell line was stained with the second layer FITC conjugate alone. One of the monoclonal antibodies (38.13) was raised in rat and hence

second layer FITC-conjugated goat anti-rat IgG was used (Dako, diluted 1:50).

12.2 Analysis of staining

Analysis of staining with monoclonal antibodies was generally carried out on an EPICS PROFILE flow cytometer (Coulter Electronics). Expression of cell surface molecules was determined by recording the mean linear fluorescence (FL) of 5000 cells in the presence of antibody. To correct for slightly differing cell sizes between cell lines the mean fluorescence intensity/size (FL/FS) ratio was calculated. Cell size was measured by forward light scatter (FS) and is proportional to cell volume. On the EPICS PROFILE FL/FS is calculated automatically for each cell.

Staining with some antibodies was analysed on a EPICS C SYSTEM fluorescence activated cell sorter (FACS). In these cases the mean FL for 25000 cells was recorded and normalised for size using the mean FS of the entire population of cells according to the following formula:

$$\frac{\text{Mean linear FL of 25000 cells}}{\text{Mean FS of 25000 cells}}$$

On each flow cytometer a bit map was used to exclude small dead cells, debris and clumped cells. All cell lines tested had unimodal size distributions.

Details of antibodies used in these experiments are given in chapter V (section 8).

13. SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS AND IMMUNOBLOTTING

13.1 Extraction and preparation of protein samples

Cells were washed twice in ice cold PBS, and the pellet resuspended in protein sample buffer [100mM Tris (pH 6.8), 20% (v/v) glycerol, 1% (v/v) 2-mercaptoethanol, 1% (v/v) SDS, 0.01% bromophenol blue] (40µl per 10^6 cells). The lysate was transferred to a sterile 1.5ml microfuge tube and DNA sheared

by sonicating (3 x 10 seconds, 14 microns). The lysates were centrifuged at 13400 x g in a microfuge to pellet cellular debris and the supernatant transferred to a fresh tube. Following heating to 100°C for three minutes the lysates were loaded onto a SDS-polyacrylamide gel. If samples were not used immediately they were stored at -20°C and heated to 100°C for 3 minutes prior to use.

13.2 SDS-polyacrylamide gel electrophoresis

The method used is an adaptation of that first described by Laemmli (1970). Polyacrylamide gels (section 13.7) were prepared between glass plates (20cm x 19cm) separated by 1.5mm spacers. 30ml of the main gel mix was poured between the plates and left to polymerize at room temperature. Polymerisation generally took 30-45 minutes. On top of the polymerised main gel a 5% polyacrylamide `stacking' gel was poured and a 'comb' inserted to form wells 7mm wide. The gels were run in vertical, discontinuous buffer, gel tanks (Medical Physics, Royal Postgraduate Medical School, London) containing 25mM Tris, 192mM glycine, 0.1% (w/v) SDS in both upper and lower reservoirs. Gels were electrophoresed at a constant current of 35mA through the stacking gel, and the current increased to 40mA for electrophoresis through the main gel.

13.3 Transfer to nitrocellulose

Transfer of electrophoresed protein to nitrocellulose (western blotting) was essentially as described by Burnette (1981). After electrophoresis the gel was immersed in transfer buffer (25mM Tris, 192mM glycine, 20% (v/v) methanol) containing 0.1% (v/v) SDS for 30 minutes at room temperature with gentle rocking. Protein was transferred to 0.45μm nitrocellulose (Schleicher & Schuell) using electrophoresis (Trans-blot cell, Bio-Rad Laboratories) in transfer buffer cooled to 4°C. Transfer buffer was kept at 4°C using a circulating water cooling system (Multitemp II, Pharmacia LKB Biotechnology). Electrophoresis was at a constant voltage of 48V overnight.

13.4 Immunoblotting.

Following transfer of protein the nitrocellulose filter was rinsed for 5 minutes in PBS/Tween (PBS containing 0.05% Tween 20). Unused protein binding sites were 'blocked' by incubating for 40 minutes at room temperature in PBS/Tween/Milk [PBS/Tween containing 2% (w/v) powdered skimmed milk]. The primary antibody, diluted in PBS/Tween/Milk, was added and incubated for 4-5 hours at room temperature, or overnight at 4°C, with gentle shaking. The

blot was washed for 5 x 15 minutes in PBS/Tween and bound antibody visualised using either the peroxidase (section 13.5) or ^{125}I-protein A (section 13.6) development sytem.

13.5 Visualisation of bound antibody using peroxidase conjugated antibody.
Following incubation with the primary antibody and subsequent washes, peroxidase-conjugated goat anti-human IgG (Nordic), diluted 1:1000 in PBS/Tween/Milk, was added. After a 30 minute incubation at room temperature, the blot was washed for 5 x 15 minutes in PBS/Tween and rinsed briefly in PBS. 50ml PBS containing 0.03% (w/v) $CaCl_2$, 0.03% (w/v) ammonium nickel sulphate, 50mg 3,3-diamino benzidine tetrahyrochloride (DAB) (Sigma) and 0.0034% (v/v) H_2O_2 was added. Colour reaction was halted by rinsing with a large volume of water, and the blot dried between 2 pieces of 3MM paper (Whatman).

13.6 Visualisation of bound antibody using ^{125}I labelled protein A.
For detection of some proteins a more sensitive detection method, using ^{125}I-labelled protein A was used (Rowe, M., *et al.*, 1987a). Following incubation with primary antibody and washing with PBS/Tween, rabbit anti-mouse IgG antibody (Dako), diluted 1:1000 in PBS/Tween/Milk, was added. After a 60 minute incubation at room temperature with gentle shaking, the blot was washed for 5 x 15 minutes in PBS/Tween and 10ml PBS/Tween/Milk containing 1μCi affinity purified ^{125}I-labelled protein A (>30mCi/mg, Amersham International) added. Incubation was for 2-3 hours at room temperature with gentle shaking. The blot was washed in PBS/Tween as before, dried between 2 sheets of 3MM paper, covered in plastic film ('Saran-Wrap', Dow Chemical Co.) and exposed to pre-flashed X-ray film for 1-7 days, depending on intensity of signal.

13.7 Composition of gels

Polyacrylamide gels for protein electrophoresis were made up as follows (all volumes are in ml).

	5%	7.5%	10%
Acrylamide*	1.67	7.5	10
1M Tris (pH 6.8)	1.25	-	-
1M Tris (pH 8.8)	-	11.2	11.2
10% (v/v) SDS	0.1	0.3	0.3
10% (w/v) ammonium persulphate	0.05	0.1	0.1
TEMED	0.01	0.02	0.02
ddH_2O	7.0	8.7	11.2

* Stock acrylamide was made up of 30% acrylamide, 0.8% bis-acrylamide, and stored in the dark at 4°C for a maximum of 4 weeks.

14. ENZYME-LINKED IMMUNOSORBENCE ASSAY (ELISA) FOR C-MYC

The ELISA method used for detection of c-Myc protein is essentially as described by Moore *et al* (1987).

14.1 Preparation of cell lysates

Cells were washed twice in ice-cold PBS containing 0.1% (w/v) sodium azide, by centrifugation at 500 x g for 5 minutes at 4°C. The cells were counted, pelleted by centrifugation and resuspended in lysis buffer (5 x 10^7 cells/ml) (TBS containing 1% (w/v) SDS, 50mM DTT, 1% aprotinin (Sigma), 0.5mM PMSF). The samples were sonicated (3 x 10 secounds, 14μm), heated to 100°C for 3 minutes and iodoacetamide (Sigma) added to 100mM. Following a 30 minute incubation on ice the samples were diluted with 9 volumes TBS containing 1% (v/v) NP40, 1% (v/v) aprotinin and 0.5mM PMSF. Lysates were stored in 1ml aliquots in liquid nitrogen.

14.2 ELISA

ELISA plates were prepared by adsorbing a polyclonal rabbit pan-myc antibody (anti-Xmyc-1) (Moore *et al.*, 1987) onto flat bottomed ELISA plates (Immulon II, Dynatech). This was achieved by adding antibody (5μg/ml in 100mM sodium

bicarbonate pH 9.6) to the ELISA plates and leaving overnight at room temperature in a moist chamber. The plates were washed twice in TBS and incubated for 30 minutes at room temperature with TBS containing 2% (w/v) dried skimmed milk. After washing once with TBS 100µl of protein lysates were added and incubated for 2 hours at room temperature. The plates were washed twice with TBS and 100µl of alkaline phosphatase conjugated mouse monoclonal anti-C-myc antibody added (Myc1-3C7, 3µg/ml in TBS containing 4% milk and 0.5% Tween 20) (Evan *et al.*, 1985). After a 30 minute incubation at room temperature the plates were washed 6 times in 'AMPAK wash solution' (IQ [Bio] Ltd). Conjugate bound to captured c-Myc protein was detected using a commercially available amplification system (AMPAK, IQ [Bio] Ltd). 100µl 'substrate solution' was added and left for 45 minutes at room temperature. To this, 50µl of 'amplifier' was added and the colour reaction halted after 5 minutes by the addition of 25µl 0.5M HCl. The colour reaction was quantified by measuring absorbence at 492nm on an automated plate reader (Flow Laboratories). Bacterially produced c-Myc protein (Watt *et al.*, 1985) was titrated to allow a standard curve to be constructed from which amounts of c-Myc protein could be calculated for the various cell lines. As a negative control lysis buffer diluted with 9 volumes of TBS (containing 1% (v/v) NP40, 1% (v/v) aprotinin and 0.5mM PMSF) was used.

All reagents and materials were gifts from Dr G. Evan and Dr T. Littlewood, Imperial Cancer Research Fund, London, UK.

15. CELLULAR GROWTH CHARACTERISTICS

15.1 Morphology

Morphology of cell lines was examined by placing a 10µl aliquot of cells in normal growth medium on a glass slide and covering with a coverslip. Cells were examined on a light microscope (Leitz Diaplan) at x600 magnification.

15.2 Growth pattern

Growth pattern of the various cell lines was assessed by plating out cells in single cell suspension at a density of 2 x 10^5/ml in normal growth medium. One ml of each cell line was plated out in 15mm, 24 well, tissue culture plates, and left at 37°C for 2 hours before being examined on an inverted microscope (Nikon TMS) at x200 magnification.

15.3 Growth Kinetics

The growth kinetics for cells in normal growth medium containing 10% FCS was monitored by seeding the cells, in fresh medium, at a density of 2.5 x 10^5/ml in 50ml tissue culture flasks. Cells were left for 14-18 days and were not fed with fresh medium during this period. Triplicate aliquots of cells were removed at regular intervals and counted using trypan blue to stain non-viable cells.

15.4 Serum dependency

To examine growth of cell lines in low serum concentrations, cells were washed twice in serum-free RPMI 1640 medium and resuspended to a density of 5 x 10^4/ml in RPMI 1640 medium containing 1% FCS. 200µl aliquots (10^4 cells) were added to each of 3 replica wells in a 96 well round-bottomed microculture plate. Cells were left for 48 hours in normal culture conditions and 1µCi [methyl-^{3}H]thymidine (70-85Ci/mmol, Amersham International) added to each well. After a 4 hour incubation with tritiated thymidine, the cells were harvested as described earlier (section 1.4), and incorporation of thymidine measured by counting on a beta particle counter.

16. ASSAYS FOR A TRANSFORMED PHENOTYPE

16.1 Colony formation in soft agar

For each cell line 4 x 10^4 cells were suspended in 2.5ml RPMI 1640 medium plus 20% FCS, 0.25ml 5x RPMI, 1ml 3% agarose (SeaPrep agarose, FMC BioProducts). 1ml aliquots were added to duplicate 15mm wells of a 24 well tissue culture plate and placed at 4°C for 10 minutes to allow the agarose to gel. The cultures were incubated in a humidified 37°C incubator in the presence of 5% CO_2 for 10 days and cultures were examined regularly for signs of colony formation. A colony was classified as a clump of 8 cells or more.

Stock agarose was made up by adding 1.5g agarose to 50ml ddH_2O and autoclaving for 15 minutes to sterilise. Before use, the agarose was heated to

60°C to melt and equilibrated to 37°C. 5x RPMI was made up as follows:

12.5ml 10x RPMI concentrate (Flow Laboratories)
1.25ml L-glutamine (29mg/ml)
0.25g sodium bicarbonate
12.5ml FCS

16.2 Tumour production in nude mice

Tumour production in nude mice was assessed essentially as described by Lombardi, *et al.* (1987). 2.5 x 10^7 cells were washed twice in PBS and resuspended in 1ml PBS. 5 x 10^6 cells (0.2ml) were innoculated subcutaneously into each of 5 nude mice. The mice were 4-6 week old, female, athymic nude mice. The mice were from an outbred litter, obtained from the National Institute for Medical Research, London. Animals were monitored for up to 8 weeks. Inoculation of the mice was carried out, under licence, by Dr. P. Smith or Dr. M.J. Allday (Royal Postgraduate Medical School, London).

17. COMMON MARMOSETS - HAEMATOLOGY AND SEROLOGY

Total white cell counts (WCC) were calculated by diluting the peripheral blood 1:10 in white cell fluid [2% (v/v) glacial acetic acid and 0.05% (w/v) crystal violet]. Counting was performed using a haemocytometer (Weber Improved Neubauer counting chamber), on a light microscope at x400 magnification. Slides for differential blood counts were made by applying a thin film of blood to a glass slide, air drying and fixing in methanol for 3 minutes at room temperature. Nucleated cells were visualised by adding Giemsa stain for 3 minutes, washing in ddH_2O and air-drying. Differential cell counts were performed by Dr. I. Reilly (Dept. of Haematology, Royal Postgraduate Medical School, London).

For total white cell counts (WCC) and differential blood cell counts peripheral blood was collected in sterile plastic tubes containing preservative free heparin. For EBV-specific serology 0.5ml of peripheral blood was allowed to clot at room temperature in a 1.5ml microfuge tube, centrifuged (3350 x g) and the serum collected and stored at -20°C until tested. Blood was collected from the marmosets by Dr. N. Wedderburn (Royal College of Surgeons, London).

18. IMMUNOPEROXIDASE PHENOTYPING OF PERIPHERAL MONONUCLEAR CELLS FROM COMMON MARMOSETS

18.1 Antibodies

Details of the antibodies used in the analysis of cell surface antigen expression on peripheral blood mononuclear cells are presented in Table 17

18.2 Staining of cells

Cells were prepared by applying a thin film of blood to a glass slide, air drying and fixing in methanol for 3 minutes at room temperature. The method used for phenotyping cells was the peroxidase anti-peroxidase method, essentially as described by van Noorden (1986). Endogenous peroxidase was blocked by immersion, for 3 minutes, in methanol containing 0.3% H_2O_2. Cell preparations were washed for 5 minutes in PBS and incubated for 60 minutes at room temperature with the primary antibodies (table 17) diluted in PBS. The slides were washed for 2 x 5 minutes in PBS and rabbit anti-mouse Ig (Dako) diluted 1:20 in PBS added. Following a 60 minute incubation at room temperature the slides were washed as before. Mouse peroxidase anti-peroxidase (Dako, diluted 1:100 in PBS) was added and the slides incubated for a further 45 minutes at room temperature. The slides were washed as before, immersed in DAB (1mg/ml) for 2 minutes and H_2O_2 added (to 0.03%). After developing for 3 minutes the slides were washed for 5 minutes in running water and counterstained in Meyers haematoxylin for 2 minutes. After a further 30 minute wash in running water the slides were sequentially immersed, for 30 seconds each, in 70% ethanol, 95% ethanol, acetone and xylene. The slides were then mounted under a coverslip using a DPX mounting solution, and examined under a light microscope at x400 magnification.

Table 17

Monoclonal antibodies used in the analysis of cell surface molecule expression in common marmosets

Antibody	Designation	Source	Reactivity	Reference
T11	CD2	Dako	T	Howard *et al.*, 1981
TO15	CD22	Dako	B	Nadler 1985
L26	ND[1]	Dako	B	Ishii *et al.*, 1986
1B5	HLA DR alpha	Dako	B, act. T, M	Adams *et al.*, 1983
LeuM1	CD15	J A Thomas[2]	M	Hanjan *et al.*, 1982

All are mouse monoclonal antibodies.

1 ND - not designated

2 Imperial Cancer Research Fund, London, UK

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APPENDIX

RELEVANT PUBLICATIONS

Hotchin, N.A., Allday, M.J., Crawford, D.H. (1990). Deregulated c-*myc* expression in Epstein-Barr virus immortalized B-cells induces altered growth properties and surface phenotype but not tumorigenicity. Int. J. Cancer, *45*, 566-571.

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DEREGULATED C-*myc* EXPRESSION IN EPSTEIN-BARR-VIRUS-IMMORTALIZED B-CELLS INDUCES ALTERED GROWTH PROPERTIES AND SURFACE PHENOTYPE BUT NOT TUMORIGENICITY

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Endemic Burkitt's lymphoma (eBL) is characterized by the presence of Epstein-Barr virus (EBV) and a chromosomal translocation which results in deregulation and constitutive expression of the c-*myc* proto-oncogene. In order to examine the role played by activation of c-*myc* in determining the eBL phenotype, we have introduced into EBV-immortalized lymphoblastoid cells (LCL) plasmids which permit constitutive expression of c-*myc*. The resulting cells show a reduced serum dependence, reduced homotypic cell aggregation, and changes in surface characteristics. In particular, levels of the cell adhesion molecule, LFA-1, are greatly reduced. However, the cells continue to express all the EBV latent antigens associated with the LCL phenotype and they remain non-tumorigenic. These results suggest that, whilst constitutive expression of c-*myc* may contribute to the malignant phenotype, it is insufficient to induce tumorigenicity.

Endemic Burkitt's lymphoma (eBL) is an aggressive, monoclonal B-cell lymphoma, which occurs at a relatively high frequency in children from Central Africa and Papua New Guinea. Endemic BL is characterized by the presence of EBV and a chromosomal translocation involving the c-*myc* proto-oncogene on chromosome 8 and immunoglobulin loci on chromosome 14, or more rarely chromosomes 2 or 22. This translocation results in constitutive, unregulated expression of c-*myc* (Cory *et al.*, 1986).

Endemic BL cells are highly tumorigenic, producing tumours in nude mice and growing in soft agar (Nilsson *et al.*, 1977). Morphologically, the cells have a regular spherical appearance and grow in single-cell suspension when cultured (Nilsson and Klein, 1982). Expression of EBV proteins in eBL is highly restricted with only the EBV nuclear antigen, EBNA-1, being detected (Rowe *et al.*, 1987*b*). Endemic BL cells are also characterized by the absence, or low-level expression, of cell-adhesion molecules, lymphocyte-function-associated antigen-1 (LFA-1, CD11a, CD18), LFA-3 (CD58) and intercellular adhesion molecule 1 (ICAM-1, CD54) (Patarroyo *et al.*, 1988; Gregory *et al.*, 1988). These molecules are thought to be of fundamental importance in cellular interactions within the immune system, mediating, in particular, B-cell/B-cell and B-cell/T-cell adhesion (Springer *et al.*, 1987).

In contrast, *in vitro* EBV-immortalized lymphoblastoid cell lines (LCL), in which the c-*myc* translocation is not present, are non-tumorigenic (Nilsson *et al.*, 1977). The cells are irregular in shape and typically grow in tight clumps in culture (Nilsson and Klein, 1982). They express the full range of EBV latent antigens; that is, EBNA 1–6 and the latent membrane protein (LMP) (Dillner and Kallin, 1988; Allday *et al.*, 1988). In addition, they express high levels of LFA-1, LFA-3 and ICAM-1 (Gregory *et al.*, 1988). Expression of LMP in EBV-negative BL cells results in an increase in the expression of cell adhesion molecules (Wang *et al.*, 1988).

The c-*myc* gene encodes a nuclear protein whose precise function is not known. High-level expression is associated with proliferating cells (Kelly *et al.*, 1983), whereas quiescent or differentiating cells express very low levels of c-*myc* RNA and *myc* protein (Westin *et al.*, 1982; Moore *et al.*, 1987). In normal cells regulation of c-*myc* expression is complex, probably involving both transcriptional and post-transcriptional mechanisms, including autosuppression by *myc* protein (Leder *et al.*, 1983; Kelly and Siebenlist, 1986; Bentley and Groudine, 1988). Studies on transgenic mice, in which the c-*myc* gene was linked to the immunoglobulin μ or k enhancer, showed a high incidence of B-cell lymphomas (Adams *et al.*, 1985). However, the monoclonality of these tumours and the length of time they took to develop suggested that constitutive expression of c-*myc* alone was insufficient to induce tumorigenicity. Indeed, recent work with transgenic mice has shown that, whilst c-*myc* can act synergistically with the v-H-*ras* and v-*raf* oncogenes to induce pre-B-cell tumours, c-*myc* alone is not sufficient (Alexander *et al.*, 1989). In contrast, Lombardi *et al.* (1987) reported that constitutive expression of c-*myc* was sufficient to induce a tumorigenic phenotype in 2 EBV-immortalized LCLs.

In order to determine what role constitutive expression of c-*myc* plays in determining the eBL phenotype, and to re-examine the role of c-*myc* in tumorigenicity in LCLs, we have stably introduced plasmids expressing c-*myc* into an LCL. We report that constitutive expression of c-*myc* results in phenotypic changes and enhanced growth characteristics, but not necessarily tumorigenicity. Although EBV gene expression was unaffected, decreased expression of LFA-1 in cells constitutively expressing c-*myc* was observed. These results suggest that *myc* may act to suppress expression of other cellular genes.

MATERIAL AND METHODS

Cell culture

The EBV-immortalized cord-blood cell line, IB4 (King *et al.*, 1980), was cultured at 5% CO_2 in RPMI 1640 supplemented with 10% foetal calf serum (FCS), 2 mM L-glutamine, 100 IU/ml penicillin and 100 μg/ml streptomycin. The concentration of hygromycin B required to prevent cell growth was determined by adding serial dilutions of the drug to cells and staining with Trypan blue 10 days later. The concentration of hygromycin B required to cause 100% cell death in non-transfected cells was 400 μg/ml. Cloning of established transfected lines was achieved by plating cells at 10 per well in 96 round-bottomed wells containing 10^4 feeder cells in RPMI 1640 medium supplemented with 20% FCS. Feeders were obtained by irradiating peripheral blood mononuclear cells from an EBV seronegative donor.

Transfection of plasmid DNA

A plasmid construction (pHEBoSV*myc*2,3) (Lombardi *et al.*, 1987) containing the coding exons 2 and 3 of the human c-*myc* gene linked to the SV40 early region promoter was introduced into IB4 cells to mimic the deregulation of c-*myc* seen in BL. Under the influence of the SV40 promoter, normal

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regulation of the gene is lost. This plasmid also carries a gene coding for the selectable marker, hygromycin B, and the EBV origin of replication (Ori-P) allowing the plasmid to be maintained and replicated as an episome in EBV-immortalized cells. A control plasmid (pHEBo) (Sugden *et al.*, 1985) containing Ori-P and the selectable marker was also introduced into IB4 cells.

Transfection was achieved by electroporation (Gene Pulser, BioRad, Richmond, CA). Cells (10^6) were resuspended in 0.8ml RPMI culture medium, placed on ice for 10 min, electroporated and left on ice for a further 10 min. The cell suspension was diluted to 2 ml with culture medium and placed in 15-mm culture plates. Selection with hygromycin B was started after 48 hr.

S1 mapping

RNA was extracted from cells in log phase growth using the guanidinium/caesium chloride method (Maniatis *et al.*, 1982) and S1 mapping was performed using a standard method (Davis *et al.*, 1986). Total RNA (20μg) was hybridized to a ^{32}P-labelled probe generated from a 472-bp PstI/SacII fragment spanning the intron 1/exon 1 junction of c-*myc* cloned in M13. The hybridization reaction was digested with 100 units of S1 endonuclease (Boehringer, Mannheim, FRG), and run on a 8M urea, 5% polyacrylamide sequencing gel. Markers were prepared by digesting 1μg pBR322 with MspI, and end-labelling with ^{32}P.

Protein analysis

Whole-cell extracts were prepared, separated by SDS-PAGE, transferred to nitrocellulose and immunoblotted for EBNA and LMP by previously reported methods (Allday *et al.*, 1988; Rowe *et al.*, 1987*a*). A polyclonal human serum (RT, 1:100) was used to probe for EBNAs 1, 2, 3, 4 and 6 (Allday *et al.*, 1988). EBNA-5 was detected using the JF186 monoclonal antibody (MAb) (Finke *et al.*, 1987) and LMP using the MAb pool CS1–4 (Rowe *et al.*, 1987*a*) (the MAbs were a gift from Dr. M. Rowe).

Protein extracts from 2×10^5 cells were assayed for levels of *myc* using a previously described ELISA assay (Moore *et al.*, 1987) which allows accurate quantitation of protein levels. A calibration curve, produced using serial dilutions of *myc* protein expressed in *E. coli*, allows results to be expressed as molecules per cell.

Growth characteristics and tumorigenicity

The growth pattern of the transfected cells was determined by dispersing 2×10^5 cells to single-cell suspension in 15-mm culture dishes. The cultures were photographed after 2 hr under an inverted microscope.

In order to examine growth factor requirements, cells were washed twice in serum-free medium and then resuspended in RPMI culture medium containing 1% FCS. Cells were plated out at 10^4 well in triplicate in microculture plates containing 96 round-bottomed wells and cultured for 2 days. Proliferation was measured by uptake of ^{3}H-thymidine, following a 4-hr incubation with 1μCi/well.

Growth in soft agar was determined by resuspending 10^4 cells in RPMI 1640 containing 10% FCS, L-glutamine, penicillin, streptomycin and 0.8% agarose (Seaprep, FMC, Rockland, ME). Tumorigenicity in nude mice was determined by inoculating 4- to 8-week-old female nude mice s.c. with 5×10^6 cells in 0.2ml phosphate-buffered saline (PBS). The mice were kept in an isolator for 8 weeks and examined weekly for appearance of tumours.

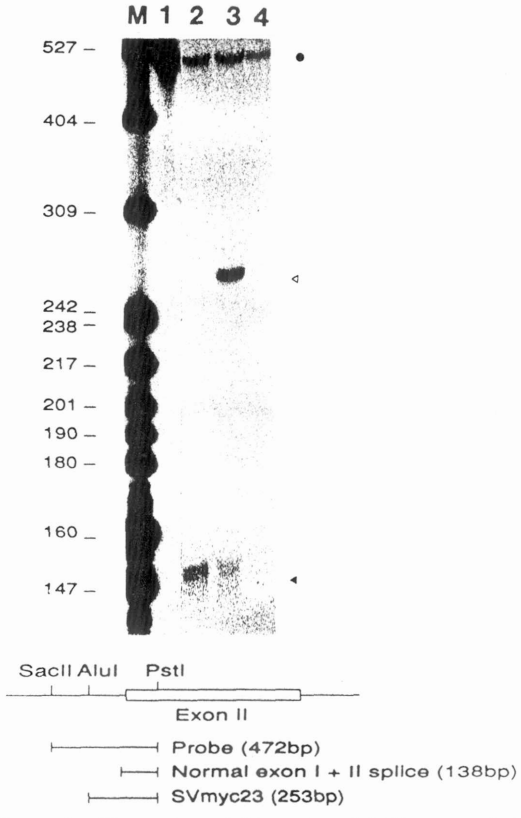

FIGURE 1 – S1 mapping of 20μg of total RNA extracted from IB4-HEBo (lane 2) and IB4-*myc*23 (lane 3). Yeast tRNA (lane 4) and probe alone (lane 1) were run as controls. Positions of plasmid-derived (◁) and endogenous (◀) transcripts are indicated as well as unhybridized probe (●).

Expression of cell-adhesion molecules

Expression of cell-adhesion molecules was quantified by indirect immunofluorescence staining followed by analysis on a fluorescence-activated cell sorter (FACS) (EPICS CS, Coulter, Hialeah, FL). Cells (5×10^5) were washed twice in PBS containing 1% BSA and 0.05% sodium azide. Mouse IgG MAb (50μl) at saturating concentration was added and the cells were left for 30 min. The cells were washed 3 times in PBS/BSA/azide, and 50μl fluorescein-conjugated goat anti-mouse IgG (Sigma, St. Louis, MO, 1:100) were added. After a further incubation for 30 min the cells were washed twice with PBS/BSA/azide, fixed in PBS containing 2% FCS and 2% paraformaldehyde for 5 min and finally washed in PBS/BSA/azide. All stages were carried out on ice and all dilutions made in PBS/BSA/azide.

TS1/22, TS2/9 MAbs (Sanchez-Madrid *et al.*, 1982) and RR-1 (Rothlein *et al.*, 1986*a*) (all gifts from Dr. T. Springer) were used to stain for LFA-1 (α-chain), LFA-3 and ICAM-1, respectively. The mean linear fluorescent intensity for 2.5×10^4 cells was calculated by staining with and without specific MAb and subtracting the background value from the positive fraction. To correct for differences in cell size between cell lines, results were expressed as a fluorescence/size ratio. The mean size of the cell population was measured by forward light scatter, and is proportional to volume.

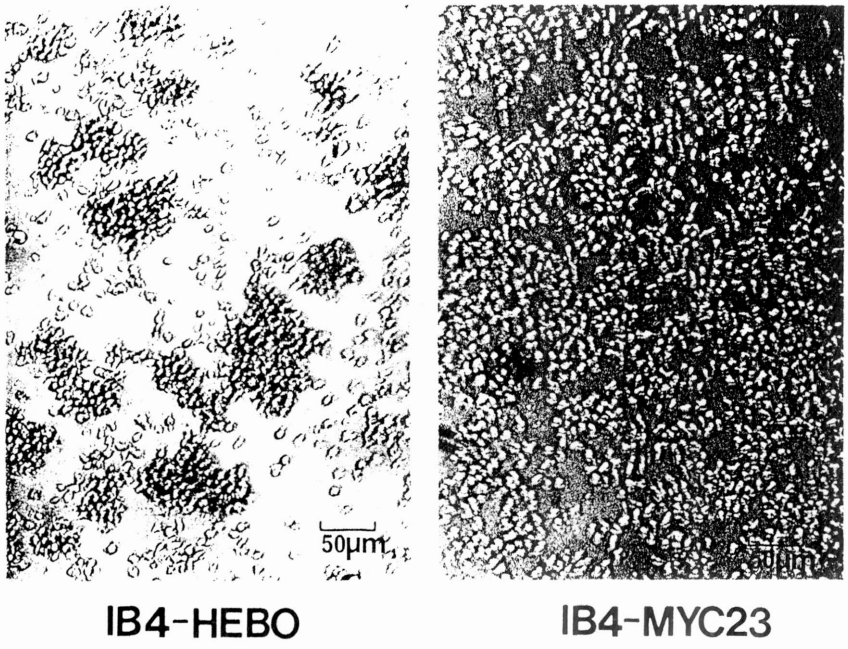

FIGURE 2 – Growth pattern of IB4-HEBo and IB4-*myc*23 cells; 2×10^5 cells were dispersed to single-cell suspension, left for 2 hr at 37°C and photographed under an inverted microscope.

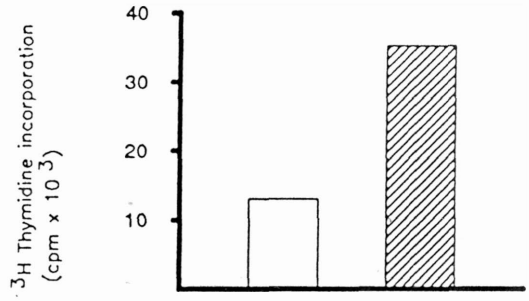

FIGURE 3 – Uptake of ^{3}H-thymidine in IB4-HEBo (□) and IB4-*myc*23 cells (▨) after 48 hr culture in 1% FCS.

TABLE I – TUMORIGENICITY OF POLYCLONAL IB4-HEBo AND IB4-*myc*23 CELL LINES AS DETERMINED BY GROWTH IN SOFT AGAR AND PRODUCTION OF TUMOURS IN NUDE MICE. THE BL CELL LINE RAJI WAS INCLUDED AS A POSITIVE CONTROL

	Tumorigenicity of transfected cell lines	
Cell line	Growth in soft agar	Tumours in nude mice
Raji	+	5/6
IB4-HEBO	–	0/5
IB4-MYC23	–	0/5

RESULTS

Establishment of cell lines

IB4 cells were successfully transfected with pHEBo and pHEBoSV*myc*2,3. Optimal transfection efficiency of 5–10% was obtained using a voltage of 0.4kV and a capacitance of 500μFd. Selection with hygromycin B (0.4mg/ml) was started after 48 hr and resistant cells were expanded and maintained with drug selection. The resultant cell lines were designated IB4-HEBo and IB4-*myc*23. The clonal nature of the parent IB4 cell line was reflected by the fact that both transfected cell lines were uniformly immunoglobulin λ light-chain-positive (results not shown).

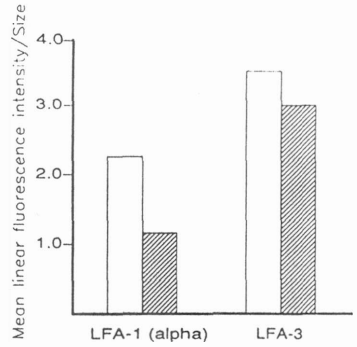

FIGURE 4 – Flow cytometric analysis of cell-adhesion molecule expression in polyclonal IB4-HEBo (□) and IB4-*myc*23 (▨) cells. Results are expressed as the mean linear fluorescent intensity/size ratio of 2.5×10^4 cells, using mouse MAbs to stain for LFA-1 (α-chain) and LFA-3.

Transcription of c-myc

To determine whether levels of c-*myc* mRNA were increased in IB4-*myc*23, and to compare expression of endogenous and exogenous c-*myc*, S1 mapping of total RNA from IB4-HEBo and IB4-*myc*23 was performed. A considerable increase in levels of c-*myc* mRNA from IB4-*myc*23 cells in relation to control IB4-HEBo cells was observed (compare Fig. 1, lanes 3 and 2). This increase in mRNA was entirely due to transcription from pSV*myc*2,3 (◁) with endogenous c-*myc* transcription (◀) apparently partially suppressed. These data are consistent with previous reports of autosuppression of c-*myc* (Adams *et al.*, 1985; Lombardi *et al.*, 1987). The increase in c-*myc*

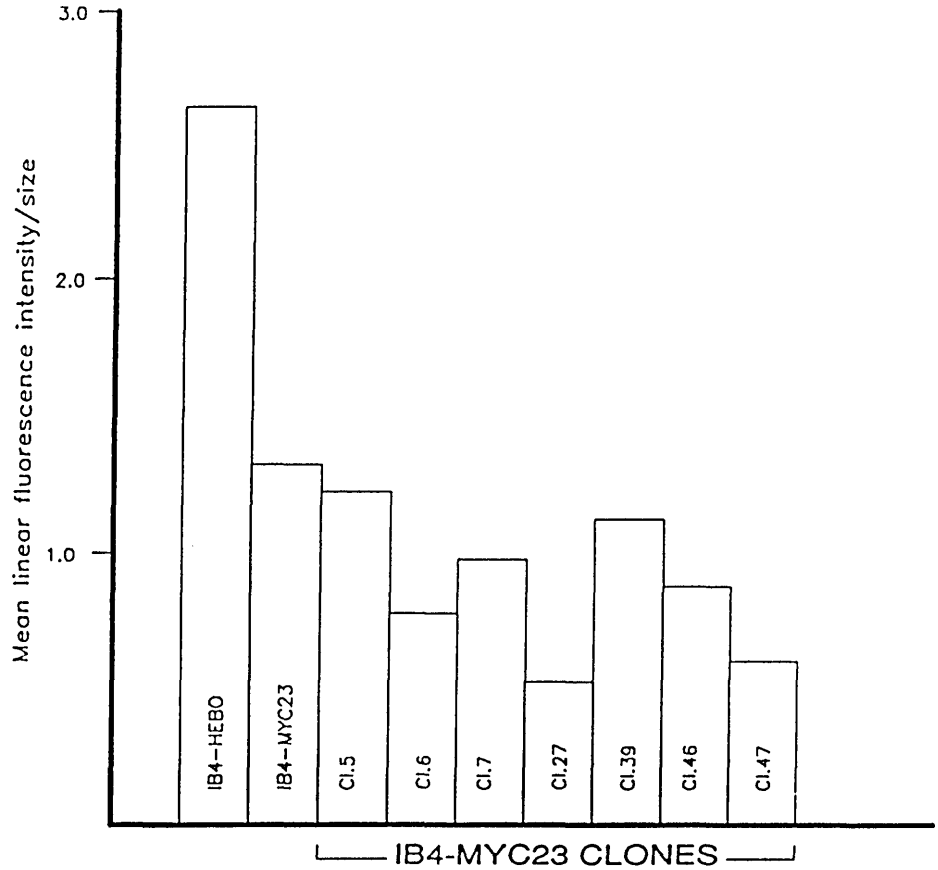

FIGURE 5 – Flow cytometric analysis of LFA-1 (α-chain) expression in 7 IB4-*myc*23 clones and the IB4-HEBo control cell line, using the TS1/22 MAb. Results are expressed as the mean linear fluorescence intensity/size ratio of 2.5×10^4 cells.

mRNA was reflected in increased levels of c-*myc* protein (see below).

Growth characteristics and tumorigenicity

Morphologically, the *myc*-transfected cells appeared unchanged; however they did show a marked reduction in homotypic adhesion compared to the control IB4-HEBo cells which grew in large, tightly packed clumps typical of the parent IB4 cell line and other LCLs (Fig. 2).

Proliferation of IB4-*myc*23 cells in 1% FCS was increased when compared to IB4-HEBo, as judged by measuring the incorporation of ^{3}H-thymidine (Fig. 3).

Neither of the cell lines grew in soft agar, nor did they produce tumours in nude mice. This contrasted with the BL cell line Raji which demonstrated high cloning efficiency in soft agar and produced tumours in nude mice after 4 weeks (Table I).

Expression of cell-adhesion molecules

Flow cytometric analysis revealed a significant decrease in expression of the α-chain of LFA-1 in the *myc*-transfected cells (Fig. 4). This finding was reproducible with cells of different passage number and on various independently established IB4-*myc*23 cell lines.

Expression of LFA-3 was slightly decreased in IB4-*myc*23 cells, but not significantly so (Fig. 4). Neither IB4-HEBo, IB4-*myc*23 nor the parent IB4 cell line expressed detectable levels of ICAM-1 (data not shown).

To examine these findings further, the IB4-*myc*23 cell line was cloned and expression of LFA-1 was analysed by flow cytometry. All 7 hygromycin-B-resistant clones obtained expressed greatly reduced levels of LFA-1 relative to the control IB4-HEBo (Fig. 5).

EBV gene expression

Immunoblot analysis revealed no detectable difference in expression of EBNAs 1, 2, 3, 5 or 6 between the 2 cell lines (Fig. 6*a,b;* compare track 1 with tracks 2 and 3). EBNA 4 is not expressed in the parental IB4 cells (Allday *et al.*, 1989). Both cell lines expressed LMP, and no significant difference in expression was detected by immunoblotting (Fig. 6*c*, compare track 1 with tracks 2 and 3). Similarly, no detectable difference in expression of the EBNAs or LMP was detected in the 7 IB4-*myc*23 clones (Fig. 6*a,b,c;* tracks 4–10).

The lytic cycle antigens—viral capsid antigen (VCA) and the early antigen complex (EA)—were not detectable by indirect immunofluorescence (results not shown), indicating that latency was strictly maintained.

Myc *protein expression*

Results obtained from the ELISA assay demonstrate an approximately 3-fold increase in the amount of *myc* protein in the

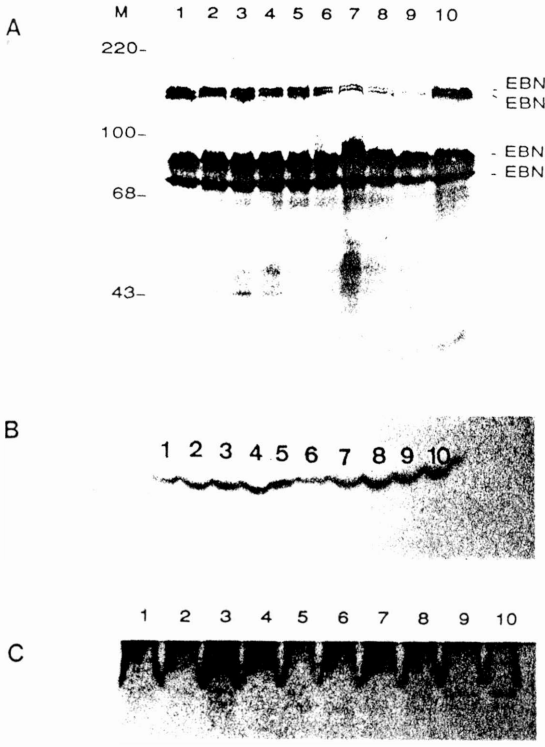

FIGURE 6 – Immunoblot analysis of EBV latent gene expression. Proteins from 10^6 cells were separated on 7.5% (EBNAs 1, 2, 3, 4 and 6) and 10% (EBNA-5 and LMP) SDS-polyacrylamide gels, transferred to nitrocellulose and probed with: *(a)* serum RT specific for EBNAs 1, 2, 3, 4 and 6; *(b)* JF186, anti-EBNA-5 MAb; *(c)* CS1–4, anti-LMP MAb pool. Lane 1: IB4-HEBo; lanes 2 and 3: polyclonal IB4-*myc*23; lanes 4–10: IB4-*myc*23 clones.

polyclonal IB4-*myc*23 cells in relation to the control line, IB4-HEBo (Table II). Similar increases in *myc* were observed in the cloned IB4-*myc*23 cell lines although there was considerable variation between the clones (compare, for example, clones 46 and 47). Interestingly, neither the polyclonal IB4-*myc*23 cell line nor the derived clones attained the levels of protein expression seen in the BL cell line Raji.

DISCUSSION

We have shown that constitutive expression of the c-*myc* proto-oncogene in EBV-immortalized lymphoblastoid cells can induce phenotypic change. Notably, expression of the cell-adhesion molecule LFA-1 is markedly reduced. The mechanism by which this occurs is not known; however, there are at least 2 possible explanations. *Myc* may suppress transcription of the LFA-1 gene in a manner analogous to the autosuppression of endogenous c-*myc* seen in this and other studies (Adams *et al.*, 1985; Lombardi *et al.*, 1987). Alternatively, *myc* may act at the post-transcriptional level to prevent expression of LFA-1 at the cell surface. We are currently investigating the precise mechanism of control.

TABLE II – *myc* PROTEIN EXPRESSION IN IB4-HEBo, POLYCLONAL IB4-*myc*23, IB4-*myc*23 CLONES AND RAJI CELLS. RESULTS FROM 2×10^5 CELLS ARE EXPRESSED AS MEAN NUMBER OF *myc* MOLECULES PER CELL.

Cell line	*Myc* molecules per cell
IB4-HEBo	1,140
IB4-*myc*23 (polyclonal)	3,660
IB4-*myc*23 clone 5	2,580
IB4-*myc*23 clone 6	1,980
IB4-*myc*23 clone 7	2,580
IB4-*myc*23 clone 27	3,360
IB4-*myc*23 clone 39	3,780
IB4-*myc*23 clone 46	4,300
IB4-*myc*23 clone 47	1,980
Raji	13,850

LFA-1 plays an important role in homotypic B-cell interactions (Mentzer *et al.*, 1985; Rothlein and Springer, 1986*b*), and the reduced aggregation seen in the c-*myc*-transfected cells may be a direct consequence of decreased LFA-1 expression. Aggregation of lymphoblastoid cells is thought to be mediated *via* the interaction of LFA-1 with its ligand ICAM-1 (Rothlein *et al.*, 1986*a*; Gregory *et al.*, 1988). However, as IB4 cells do not appear to express ICAM-1, yet still grow in clumps, it is possible that another ligand for LFA-1, such as the recently identified ICAM-2 (Staunton *et al.*, 1989), is involved in the homotypic adhesion of IB4 cells.

Another important role of LFA-1 is in mediating adhesion of cytotoxic T cells to target cells (Springer *et al.*, 1987). It has been proposed that decreased expression of this molecule may contribute to escape from immunosurveillance by tumour cells (Clayberger *et al.*, 1987). In addition, Inghirami *et al.* (1988) have proposed that lack of LFA-1 may result in a more aggressive tumour-cell phenotype. Thus, constitutive c-*myc* expression may directly contribute to the malignant nature of BL cells by causing a down-regulation in LFA-1 expression.

No detectable change in EBV gene expression was observed, suggesting that the restricted gene expression seen in BL cells is not a consequence of the translocation and consequent activation of c-*myc*. Wang *et al.* (1988) have shown that transfection of EBV-negative BL cells with a plasmid encoding LMP resulted in upregulation of LFA-1. Our results provide no evidence that the observed modulation of LFA-1 expression in IB4-*myc*23 cells is mediated by changes in levels of LMP.

The *myc*-transfected cells showed a decreased growth factor requirement as indicated by growth in reduced serum concentrations. This could well provide the cells with a growth advantage over normally regulated cells. These results are similar to those of Lombardi *et al.* (1987). However, in contrast to Lombardi *et al.*, we find that constitutive c-*myc* expression in EBV immortalized cells does not induce tumorigenicity, as determined by either growth in soft agar or tumour production in nude mice. One possible explanation for this discrepancy is that a threshold level of *myc* protein is required for induction of tumorigenicity and that the IB4-*myc*23 cells were expressing less *myc* than the lines studied previously. The BL cell line Raji, with almost 4-fold higher levels of *myc* protein than the IB4-*myc*23 line, was tumorigenic, and it is possible that the threshold lies between these 2 values. An alternative explanation is that a further event is required for tumorigenicity, for example activation of another oncogene such as *ras* which, in cooperation with c-*myc*, induces tumorigenicity in B cells (Alexander *et al.*, 1989). The clonality of the cells used in these experiments may also account for the differences between our results and those of Lombardi *et al.* The majority of LCLs are polyclonal and may contain sub-populations of cells which vary in their susceptibility to *myc*-induced transformation. We cannot rule out the possibility that the IB4 clone used in our experiments may represent a population of cells relatively resistant to complete malignant transformation by c-*myc*, although their growth characteristics are clearly altered.

In summary, cells in which expression of c-*myc* is deregulated have a decreased growth factor requirement which may result in a growth advantage over normally regulated cells, but not necessarily in a tumorigenic phenotype. In these cells,

EBV gene expression is unaffected, but the constitutive expression of exogenous c-*myc* results in reduced expression of the cell-adhesion molecule LFA-1. The mechanism by which this occurs has not yet been determined. Down-regulation of this molecule, which is important in interactions with cells of the immune system, may contribute to escape from immunosurveillence and to the aggressive nature of eBL.

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NOTE ADDED IN PROOF

Subsequent to performing the experiments detailed in this report, low level mycoplasma infection was detected in the IB4-HEBo and IB4-*myc*23 cell lines. Mycoplasma infection was not detectable when the cells were under selection with hygromycin B.

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IMMUNOHISTOLOGY OF EPSTEIN-BARR VIRUS–ASSOCIATED ANTIGENS IN B CELL DISORDERS FROM IMMUNOCOMPROMISED INDIVIDUALS

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Proliferating B cell lesions developing in a series of immunosuppressed organ transplant recipients and patients with X-linked lymphoproliferative syndrome were examined for Epstein-Barr virus and cellular gene expression using immunocytochemistry and immunoblotting techniques. Results indicate that all the lesions examined from the patients in this series expressed Epstein-Barr virus gene products that were consistent with a latent, nonproductive type of infection. No lytic cycle antigens associated with productive viral infection were detected. This pattern is similar to the viral gene expression in normal B cells immortalized by Epstein-Barr virus in vitro. The demonstration in this study of Epstein-Barr virus viral gene expression in posttransplant and X-linked proliferative syndrome B cell disorders provides important new evidence for the primary role of Epstein-Barr virus in the development of these lesions. This is in contrast to the subsidiary role that the Epstein-Barr virus has in the etiology of Burkitt's lymphoma.

In addition to infectious mononucleosis (IM)* and Burkitt's lymphoma (BL), Epstein-Barr (EB) virus is associated with a variety of B cell tumors in patients with congenital or acquired immunodeficiency (*1*). These include lymphoproliferative lesions that frequently develop as a complication of iatrogenic immunosuppression in organ transplant recipients (*2*), following infection with human immunodeficiency virus (*3*) or in individuals with a primary genetic susceptibility to EB virus infection (*2, 4*). A common factor in the development of these lesions is thought to be abnormal or deficient regulation of normal immune responses to EB virus.

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* Abbreviations: BL, Burkitt's Lymphoma; B-LCL, B lymphoblastoid cell line; CAM, cell adhesion molecule; EA, early antigen; ICAM, intercellular adhesion molecule; IM, infectious mononucleosis; LFA, lymphocyte function antigen; LMP, latent membrane protein; MA, membrane antigen; XLPS, X-linked lymphoproliferative syndrome.

EB virus infects normal resting B cells via the CD21/EB virus receptor, which is also the CR2 receptor for C3d complement binding (*5*). The virus causes polyclonal B cell proliferation in vivo and the production of permanent B lymphoblastoid cell lines (B-LCL) in vitro. In vitro immortalization of normal B cells is accompanied by the expression of viral and cellular antigens associated with various stages of B cell differentiation and activation. EB virus-induced nuclear antigens, of which up to six components (EBNAs 1–6) are now known to exist (*6–8*), appear in the initial stages of infection. EBNA-1 is important for maintaining episomal/circular viral DNA and may have additional repressive influences on the lytic cycle. EBNA-2 is thought to be involved in cellular activation and initiation of immortalization. The precise functions of the other EBNA types (including EBNA-3) have yet to be determined. Persistent expression of specific EBNA subtypes and latent membrane protein (LMP) (*7, 9*) characterizes the latent/nonproductive cycle of infection during which viral infection is maintained with continued cell proliferation but without production or release of virus. The productive/lytic cycle involving release of infectious virus particles is characterized by sequential expression of early antigen (EA), viral capsid antigen, and membrane antigen (MA) complexes (*6, 7*). The extent to which virus replication occurs in B cells and the nature of the viral-cell interaction in vivo is not known. However, the persistence of virus-infected B cells in normal individuals following primary infection clearly necessitates a precisely regulated interaction between host cells and the virus. The control of EB virus lymphoproliferation in vivo is mainly T cell-dependent (*10*), but also involves specific humoral responses (*11*) and natural killer cell activity (*12*). Deficiency of, or alteration in, these immunological mechanisms would inevitably engender varying degrees of uncontrolled B cell proliferation.

The implication of EB virus in the pathogenesis of B cell disorders in immunosuppressed individuals has been derived from pathological and serological data and more direct evidence based on immunocytochemical demonstration of EBNA (*13, 14*) and the presence of virus DNA by molecular hybridization techniques (*14, 15*). The spectrum of morphological changes exhibited by these lesions is considered to represent a distinct histopathological entity characterized by diffuse polymorphic B cell hyperplasia or diffuse polymorphic lymphoma (*1, 2, 16*). Since some of these lesions express polyclonal immunoglobulin, it is thought that they represent a hyperproliferative response induced by EB virus following a primary B cell infection or an outgrowth of latently infected cells released from normal immunoregulatory controls by ineffective or deficient T cell responses. The presence of monoclonal or multiple clonal populations in many cases demonstrates the inherent capacity for these reactive polyclonal populations to undergo clonal selection and malignant change.

One of the mechanisms by which cells may escape immunosurveillance is through loss of specific cell adhesion molecules (CAMs), which are involved in cognitive interactions between various cell types. Receptor-ligand pairing between lymphocyte function antigen-1 (LFA-1) and intercellular adhesion molecule-1 (ICAM-1) is thought to mediate the interactions between T and B lymphocytes with other cells (*17*), whereas the CD2 and LFA-3 receptor-ligand pair are primarily associated with cell adhesions involving T cells (*17*). Recent studies of EB virus-infected cells have shown that, whereas B-LCLs can express high amounts of LFA-3 and ICAM-1 and are susceptible to cytotoxic T cell activity, BL tumor cells and early BL cell lines in culture either lack or only weakly express these molecules (*18*). The relative resistance of BL cells to cytotoxic T cell activity (*18*) has led to speculations that selective CAM

TABLE 1. Clinical data

Organ transplant recipients[a]				Tumor site	Postgraft interval (days)	Specific tumor Rx[b]	Clinical response[c]
Patient	Age (years)	Sex	Graft				
1	20	M	Kidney	Kidney graft	82	Acyclovir + DXT to graft	CR by 1 week; alive
2	42	M	Kidney	R inguinal LN; tongue	183 255	CTX	CR by 18 months; alive
3	57	F	Kidney	Abdominal wall; kidney graft; liver	365	CTX	NR; dead
4	1.8	F	Heart	Small intestine	599	Acyclovir + surgical excision	CR by 3 months; alive
5	6	F	Heart	L + R tonsil	73	Acyclovir + surgical excision	CR by 3 months; alive
6	5	F	Heart/lung	L lung graft	102	Acyclovir	CR by 3 months; alive
7	23	F	Heart/lung	L lung graft	74	Acyclovir	CR by 3 months; alive
8	34	M	Heart/lung	L tonsil	155	Acyclovir + surgical excision	CR by 3 months; alive
X = linked lymphoproliferative syndrome:							
9	5	M	—	LN, spleen, liver		Prednisolone, antibiotics	Dead 13 days after presentation of disease
10	16	M	—	LN, lung, liver		Prednisolone, antibiotics	Dead 17 days after presentation of disease

[a] All patients received CsA and AZA immunosuppression at the time of grafting.

[b] Immunosuppressive agents were reduced/withdrawn in all patients; DXT = radiotherapy; CXT = (patient 2) vincristine + bleomycin; (patient 3) adriamycin, vincristine, methotrexate, etoposide, prednisolone.

[c] CR = complete response; NR = no response.

loss may be a contributory factor in allowing malignant B cells to evade immunological control.

In this study, lymphoproliferative lesions developing in a series of organ allograft recipients and patients with acute and rapidly fatal IM have been analyzed for the presence of EB virus-coded antigens, CAMs, and B cell markers functionally implicated in EB virus induced proliferation. The availability of new monoclonal antibodies to viral lytic and latent cycle antigens has provided a unique opportunity to examine the expression and cellular distribution of EB virus gene products in pathological samples using immunohistological techniques. The aim of the study was to compare the phenotypic expressions and biological behavior of the tumors in the two groups of patients and correlate these findings with known patterns of EB virus expression in BL and EB virus-immortalized B-LCL in vitro.

MATERIALS AND METHODS

Clinical data. Lymphoproliferative lesions were examined in 10 patients of whom 8 had received either renal (2 men, 1 woman, age range 20–57 years), heart (2 girls, ages 1.8 and 6 years) or heart/lung (1 male and 2 female patients, age range 5–34 years) transplants between November 1979 and October 1988, and 2 boys, aged 5 and 16 years, who had developed rapidly fatal IM-like diseases. The clinical data are shown in Table 1. HLA compatiblity between the donor-recipient pairs in the transplant group was complete in patient 2, partially matched at 1, 2, and 4 loci in patients 1, 6, and 7, respectively and showed complete mismatch at all loci in patient 3. Complete tissue typing of donor-recipient pairs was not available for patients 4, 5, and 8. All patients received immunosuppression with cyclosporin, and azathioprine, and steroids, at the time of grafting. With the exception of cases 3, 4, and 5, all patients received high-dose solumedrone for rejection episodes and additional antithymocyte globulin in cases 6 and 7. Lymphoid tumors developed between 73 and 599 days after grafting—and, with the exception of 3 cases (patients 2, 5, and 8)—these were extranodal in presentation. None of the patients except patient 5 had overt clinical symptoms indicative of IM preceding tumor diagnosis. Primary tumors within grafted organs (patients 1, 3, 6, and 7) developed asymptomatically and were identified either from surgical biopsies performed for monitoring renal graft rejection or, as in the case of two heart/lung recipients, from routine chest roentgenogram. In the other cases, tumors appeared as a palpable abdominal mass (patient 4), local lymph node enlargement (patients 2 and 8) and as generalized lymphadenopathy with respiratory obstruction (patient 5). Specific treatment consisted of immediate CsA withdrawal in all cases, short-course radiotherapy, and/or cytotoxic drugs in patients 1, 2, and 3 and surgical excision of tonsil tumors causing obstruction in patients 5 and 8. All cardiac graft recipients and one renal graft recipient (patient 1) were also treated with acyclovir. In most heart/lung recipients there was a rapid positive response to therapy with complete tumor resolution within three months or no tumor recurrence following surgical excision, and all patients remained alive with functioning grafts. In one cardiac allograft recipient (patient 4) the large abdominal mass showed slow response to initial cytotoxic chemotherapy and withdrawal of CsA. Two months after tumor diagnosis, intestinal obstruction necessitated a partial small bowel resection that contained residual tumour. However, complete tumor resolution was achieved by three months and the patient remains alive with no evidence of residual disease. Patient 2 developed a second tumor on the posterior aspect of the tongue while receiving therapy for the initial lymph node lesion. Both tumors, which responded to a change in cytotoxic regimen, had completely resolved by 18 months and the patient remains alive. In patient 1, there was a rapid response to withdrawal of CsA and treatment with radiotherapy to the grafted kidney together with acyclovir. A biopsy a week later was negative for proliferating B cells but 4 weeks later the patient underwent transplantation nephrectomy due to progressive rejection in the grafted organ. Patient 3 died 23 days

TABLE 2. Reagent panel

Antigen	Monoclonal antibody[a]	Source[a]/ reference	Specificity
EBNA-2a+b	PE2	*20*	EB virus-associated antigens
EBNA-3	T278	[b]	
VCA	F323	Seralab	
MA	H140	Seralab	
EA	1108-1	Biogenesis	
LMP	S12	*9*	
LMP pool	CS1-4	*21*	
CD45	PD7	Dakopatts	Common leukocyte and B cell antigens
CD19	B4	Coulterclone	
CD20	B1	Coulterclone	
CD22	To15	Dakopatts	
—	L26	Dakopatts	
CD21	OKB7, HB5, B2	Orthomune, Becton Dickinson Coulterclone	B (and T) cell activation antigens
CD23	MHM6	*5*	
CD38	OKT10	Orthomune	
CD77	38.13	*22*	BL antigen
CD11a (LFA-1)	TS1/22	*17*	Cell adhesion molecules
CD54 (ICAM-1)	RR1/1		
CD58 (LFA-3)	TS2/9		
HLA-ABC (monomorphic)	W6/32	Seralab	MHC antigens
HLA-A3	X1.23.2	*23*	
HLA-DR3	16.23	*24*	
HLA-DRα	1B5	Dakopatts	
HLA-DPα; DPβ	DP11.1; B7/21	*25*	
HLA-DQ	Tu22	Biotest	
CD1	NA1/34	Seralab	T cell antigens
CD2	T11	Coulterclone	
CD3	T3	Coulterclone	
CD4	T4	Coulterclone	
CD8	T8	Coulterclone	
IgM, G, A, D, k		Sigma/Dakopatts	Immunoglobulin
Cytokeratin	CAM5.2	Dakopatts	Squamous epithelium

[a] Murine mAbs were detected with commercially available labeling reagents from Dakopatts using either unconjugated rabbit antimouse Ig (dilution 1/25) with mouse monoclonal APAAP (dilution 1/20) or PAP (dilution 1/100); or biotinylated rabbit antimouse Ig (dilution 1/250) and ABC-Px (dilution 1/100). Rat mAb to CD77 antigens was labeled with goat antirat IgM-Px conjugate (dilution 1/25) obtained from Southern Biotechnology.

[b] MAbs that were kindly supplied by individual laboratories were as follows: mAbs PE2, T278 and CS1-4 from Professor A. Rickinson, Cancer Research Campaign Laboratories, Department of Cancer Studies, University of Birmingham Medical School, UK; mAb S12 from Dr. D. Thorley-Lawson, Department of Pathology, Tufts University, Boston, MA; mAb MHM6 from Professor A. McMichael, Department of Medicine, Oxford University, UK; mAbs LFA-1, ICAM-1 and LFA-3 from Professor T. Springer, Laboratory of Membrane Immunochemistry, Dana-Farber Institute, Boston, MA; mAb 38.13 from Dr. J. Weils, Laboratory of Cellular Immunobiology, Institut Gustave Roussy, Villejuif Cedex, France.

after tumor diagnosis, with evidence of widespread disease affecting the graft, liver, bone marrow, brain, and meninges at autopsy.

In the second group, both patients were previously healthy and presented with an overwhelming acute IM-like illness that was rapidly fatal despite intensive supportive therapy with prednisolone and antibiotics. At autopsy, both patients were found to have widespread lymphoproliferative lesions and a retrospective diagnosis of X-linked proliferative syndrome (XLPS) was made on the basis of their serological findings and those of close family members.

Tissues. Portions of surgical biopsies and autopsy specimens were snap-frozen and conventionally processed by formol/saline fixation and paraffin wax embedding for immunocytochemistry and routine histology.

Cell lines. The specificity of the anti-EBNA reagents was determined by immunocytochemistry and immunoblotting techniques on cytocentrifuge preparations or protein extracts of well-established B-LCL and BL cell lines. EB virus genome-positive cells included 2C9 and IB4 human B-LCLs; B958 marmoset LCL and Raji BL cell lines known to contain most EBNA components; P3HR1; AW Ramos and Daudi BL cell lines, which lack EBNA-2 expression; and DG75 EBNA-negative BL cells transfected with EBNA-2 (*19*). EB virus genome negative Ramos and BL41 cell lines were used as negative controls.

Antibodies. The panel of mAb reagents used is shown in Table 2. EB virus lytic cycle proteins VCA, EA, and MA were detected with mAbs F323, 1108-1 and H140, respectively. EBNA components were demonstrated with mAbs PE2 and T278 specific for EBNA-2 and EBNA-3, respectively, and with immune human sera containing activity to EBNA-1 (JAT), EBNA-2 (WL), EBNA-2 + EBNA-3 (WC) and EBNAs-1-4,6 (RT). LMP was detected with mAb S12 and with a pool of monoclonal antisera CS1-4. The rest of the panel was designed to characterize the proliferating neoplastic cells with respect to B cell differentiation (CD19, CD20, CD22, CD38) antigens, EB virus-associated differentiation and activation (CD21, CD23) antigens, Burkitt's lymphoma glycolipid antigen (BLA, CD77), immunoglobulin isotype (IgM,A,G,D, k and l) status; and CD11a (LFA-1), CD54 (ICAM-1), and CD58 (LFA-3) expression. The presence of class I and II major histocompatibility antigens was determined with mAbs to monomorphic HLA-ABC determinants and HLA-D region products. MAbs to appropriate polymorphic HLA determinants were available in two instances in which donor-recipient pairs were histoincompatible at the HLA-A3 or HLA-DR3 loci. Tissue typing studies were performed to ascertain the origin of the neoplastic cells in each case. Antigenic markers for immature (CD1) and subsets of mature (CD2, CD3, CD4, CD8) T cells, and cytokeratins were used to exclude a non-B cell origin of the lesions and broadly identify accompanying populations of reactive cells.

Immunohistology. Phenotyping studies were performed on 5-μm cryostat sections using the peroxidase-antiperoxidase, alkaline phosphatase anti-alkaline phosphatase or avidin-biotin-peroxidase complex (ABC-Px) techniques (*26*). Most mAbs were demonstrated on sections prefixed for 5 min in cold (4°C) chloroform:acetone (1:1) and briefly rinsed in phosphate-buffered saline, pH 7.2, prior to staining. Optimum section fixation for lytic cycle antigens EA and VCA was achieved with cold (4°C) acetone for 5 min and for BLA with neutral buffered formalin at 22°C for 5 min. All antibody incubations were performed for 60 min in a damp chamber at 22°C with intervening rinses in PBS. Visible enzyme reaction products were developed with 3'3' diaminobenzidine (Sigma, UK) for (brown) Px and naphthol ASBI phosphate (Sigma) with fast red TR salt (Raymond Lamb, UK) in veronal acetate buffer pH 9.6 containing 1 mmol levamisole for (red) AP. The reactivity patterns of all mAbs were monitored on appropriate tissue (tonsil) and EB virus genome-positive marmoset (B958) and negative (Ramos) cell line controls. EBNA proteins were detected with immune human sera using the anticomplement indirect method with Px (ACIPx test; 27) and fluorescein isothiocyanate, (ACI-FITC test [*28*]) as labels. Cryostat sections or cytocentrifuge preparations, prefixed for 5 min in equal parts of cold (−60°C) methanol:acetone and air dried, were incubated in a mixture of heat-inactivated EBNA-positive serum (dilution 1/10–1/80) and human complement (EBNA negative) serum (dilution 1/10) in complement fixation buffer (Oxoid) for 60 min at 37°C. Tissue-bound antigen was detected with rabbit antihuman C3-Px (Dakopatts; dilution 1/25) or with rabbit antihuman C3-FITC (Dakopatts; dilution 1/25). All intervening rinses were performed in complement-fixation buffer. To exclude nonspecific staining, all tissues were similarly examined with heat-inactivated EBNA-negative serum and with the anti-C3 labeling antibodies alone. Cultured cells B958, Raji, and Ramos were used as EBNA-positive and -negative controls. Finally, all immunoenzyme-stained preparations were lightly counterstained in Mayer's hemalum, coverslipped, and examined by light microscopy. Immunofluorescent preparations of EBNA were examined without counterstaining using a Zeiss epifluorescent microscope.

Serology. Acetone-fixed preparations of P3HR1 and tetradecanoylphorbol-13-acetate-induced Raji cells were used to determine serum antibody levels to VCA and EA by indirect immunofluorescence (*29*). Serum antibodies to EBNA were detected on methanol:acetone-fixed Raji cell cytocentrifuge preparations by the anticomplement immunofluorescence test. Heterophile antibodies were detected with a commerical monospot kit (Mercia Diagnostics, UK).

Protein extraction and immunoblotting. Due to the limited amount of suitable biopsy material available, these studies were only performed on one tumor sample. Frozen tissue was thawed and minced finely with a scalpel blade. Proteins were solubilized in sodium dodecyl sulfate sample buffer by dounce homogenization, sonication, and boiling. Lymphoblastoid cells 2C9 and IB4 or BL cells, AW Ramos, BL41 or P3HR1 were washed in PBS pH 7.2 and proteins solubilized by sonication and boiling in SDS sample buffer. SDS-polyacrylamide electrophoresis on 7.5% gels and Western immunoblotting with human serum RT (for EBNA species) and mAbs CS1-4 (for LMP) were performed as previously described (*8, 21*).

RESULTS

Serological data. All the patients in the transplant group, except for patients 3 and 8, showed serological evidence of active EB virus infection at a time coincident with the appearance of their tumors (Table 3). Patients 1, 5, and 7, who were previously seronegative, developed a primary EB virus infection within 84 days of grafting, indicated by either IgM and IgG or IgG antibodies to VCA and positive monospot reactions in two cases. Patients 2 and 4 also had evidence of a primary infection, although the serological pattern in patient 2 was complicated by low-but-detectable IgG antibody titers to VCA and EA near the time of grafting. These were assumed to have been passively acquired from previous blood transfusions. However, the appearance of IgM antibodies to VCA and weak monospot reactivity in this case provided more definitive evidence of seroconversion postgrafting. Although not typical of primary EB virus infection in normal individuals, the low antibody titers to EA and EBNA seen in this patient series is not uncommon in organ transplant recipients in whom rising VCA antibody levels may be the only indication of a primary infection (*30*). In Patient 6 the serological pattern of rising IgG antibody titers to VCA indicated virus reactivation and in patients 3 and 8, although the serological data were incomplete due to lack of appropriate postgrafting serum samples, the pattern was consistent with that of normal seropositive individuals. The serology of patients 9 and 10 has been previously reported in detail (*31, 32*). In both patients specific IgM and/or IgG antibody responses to VCA, together with low or absent antibody titers to EBNA were indicative of an acute primary or persistent EB virus infection. Although both patients lacked detectable antibodies to EA which is unusual for XLPS, extended family studies revealed high anti-VCA and/or EA antibody levels in the mothers of each case, suggesting carrier status for the disease.

TABLE 3. Serological data

Patient	Serum antibody titers*					Serum samples: days postgrafting Graft-tumor interval (days)	Serological pattern
	Monospot	VCA IgG	VCA IgM	EA IgG	EBNA		
1	−	<10	NT	<10	<10	Pregrafting	
	+	32	10	<10	NT	74	Primary infection
	+	32	32	<10	<10	86 (82)	
2	−	16	NT	10	NT	9	
	±	10	10	NT	NT	197 (183)	Primary infection
	−	10	10	NT	NT	205	
	NT	10	<10	NT	NT	253 (255)	
3	—	128	NT	16	10	Pregrafting	Normal seropositive
	—	128	NT	16	10	5-23 (365)	
4	NT	2500	>40	320	<10	433	Primary infection
	NT	640	20	40	80	482 (599)	
5	NT	<10	<10	<10	<10	Pregrafting	Primary infection
	NT	80	<10	<10	<10	68 (73)	
6	NT	16	NT	<10	NT	Pregrafting	
	−	32	<10	<10	10	3	Reactivation
	−	128	<10	<32	<10	93 (102)	
7	−	<10	NT	<10	<10	Pregrafting	
	−	16	NT	<10	10	3	Primary infection
	+	64	NT	NSS	NT	84 (74)	
	+	64	NT	NSS	<10	133	
8	NT	20	<10	<10	10	11	Normal seropositive
	NT	10	<10	<10	NT	19 (155)	
9	+	64	128	<10	−	—	Primary infection
10	+	128	10	<10	4	—	Primary infection

* NT: not tested; NSS: nonspecific staining, probably due to autoantibodies.

Histology. There was striking histological similarity between the lesions from the organ transplant recipients. In general these consisted of diffuse polymorphic proliferations of large cells composed of immunoblasts and/or centroblasts together with varying numbers of centrocytes, plasmacytoid, and typical plasma cells (Fig. 1). In patient 8, the predominantly plasmacytoid proliferation containing numerous large macrophages was highly reminiscent of the starry sky pattern usually associated with BL. Typically, the lesions showed extensive invasion and obliteration of surrounding normal structures and, in some cases, contained areas of focal or widespread necrosis. Mitotic figures were not prominent apart from the tumor in patient 5. In individual cases, the lesions were classified as diffuse large-cell lymphoma of predominantly centroblastic (patients 1, 2, 4, 6, and 8), centroblastic/immunoblastic (patient 5), centroblastic/centrocytic (patient 7), or undifferentiated (patient 3) type.

There were no histological differences between the tongue and lymph node lesions in patient 2 and, similarly, the autopsy samples of liver, brain, and bone marrow from patient 3 contained tumor infiltrates that were morphologically identical to the primary renal lesion. Residual tumor present in resected small bowel from patient 4 showed a more mature pattern of small lymphocytes and plasma cells compared with the immature forms present in the first biopsy. In the XLPS patients,

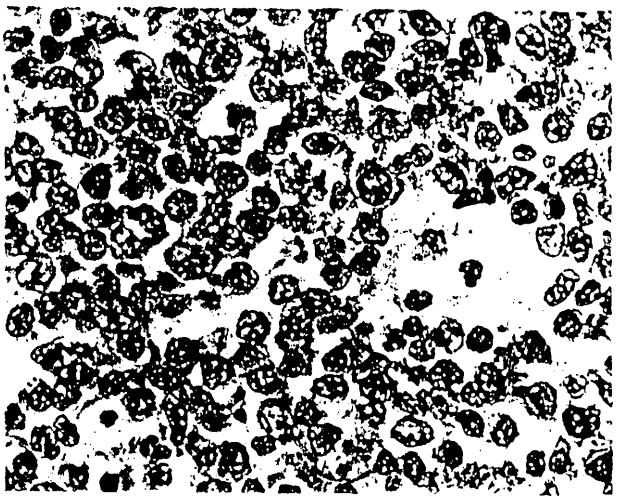

FIGURE 1. Posttransplant B cell lymphoma (patient 5) shows polymorphic tumor cell population containing immunoblasts, centroblasts, and plasmacytoid cells. (H&E staining of paraffin embedded sections; ×630.)

TABLE 4. Histology and immunophenotypes of lymphoproliferative lesions in posttransplant and XLPS patients

Patient	Tumor histology[a]	EB virus-associated antigens[b]					Ig	B cell antigens[b]								CAMs[b]		
		EBNAs 1,2,3	LMP	MA	VCA	EA		CD 45	CD 22	L26	CD 19	CD 21	CD 23	CD 38	CD 77	CD11a (LFA-1)	CD54 (ICAM-1)	CD58 (LFA-3)
1	LCL (cb)	+ + −	−	−	−	NT	−	+	+	NT	+	+	NT	−	NT	NT	NT	NT
2	LCL (cb)	+ ± +	±	−	−	NT	−	+	+	NT	−	−	+	−	NT	±	±	+
3	LCL (ud)	+ + −	±	−	−	−	IgMκ+	+	−	+	−	+	+	−	−	±	+	+
4	LCL (cb)	+ + +	−	−	−	−	Ig?κ+	+	NT	+	−	−	−	+	−	+	−	−
5	LCL (cb, ib)	+ + +	−	−	−	−	κ+λ+	+	NT	+	−	−	−	−	−	+	+	+
6	LCL (cb)	+ + −	−	−	−	NT	IgMκ+	+	+	NT	+	−	+	−	−	±	+	+
7	NHL (cb, cc)	+ + ±	−	−	−	NT	IgMκ+	+	NT	NT	−	−	+	−	NT	+	+	+
8	BLPD (cb)	+ + −	+	−	−	−	κ+λ+	+	NT	+	−	−	−	−	−	+	+	+
9	BLPD (ud)	+ − −	±	−	−	NT	κ+λ+	+	−	NT	−	−	−	+	NT	−	−	−
10	BLPD (ib, pc)	+ + ±	+	−	−	NT	κ+λ+	+	−	NT	+	−	−	+	NT	−	−	+

[a] LCL: large cell lymphoma; NHL: non-Hodgkin's lymphoma; BLPD: B lymphoproliferative disease; cb: centroblastic; cc: centrocytic; ib: immunoblastic; pc: plasmacytic; ud: undifferentiated.

[b] NT: not tested; immunocytochemical reactions scored as: positive (+), weak (±), and negative (−).

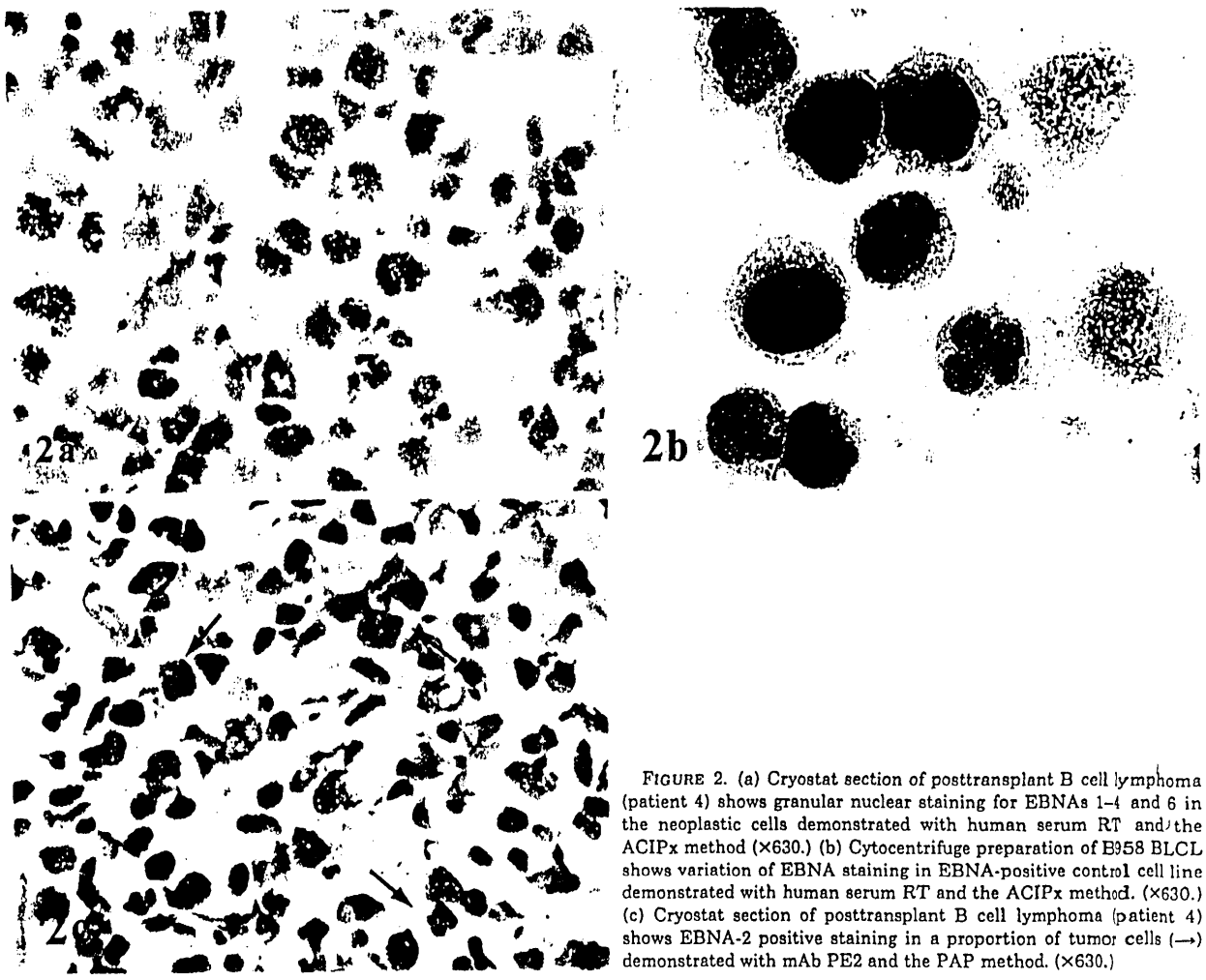

FIGURE 2. (a) Cryostat section of posttransplant B cell lymphoma (patient 4) shows granular nuclear staining for EBNAs 1–4 and 6 in the neoplastic cells demonstrated with human serum RT and the ACIPx method (×630.) (b) Cytocentrifuge preparation of B958 BLCL shows variation of EBNA staining in EBNA-positive control cell line demonstrated with human serum RT and the ACIPx method. (×630.) (c) Cryostat section of posttransplant B cell lymphoma (patient 4) shows EBNA-2 positive staining in a proportion of tumor cells (→) demonstrated with mAb PE2 and the PAP method. (×630.)

autopsy tissues from all body organs were examined. Primary and secondary lymphoid organs from patient 9 were severely lymphodepleted and contained diffuse infiltrates of large immature cells, focal necrosis, and prominent histiocytic hemophagocytosis. Similar neoplastic large-cell deposits were observed in the liver. By contrast in patient 10, diffuse polymorphic infiltrates containing immunoblasts and plasmacytoid cells were present in the lymphoid organs, liver, and respiratory tract mucosa and also formed multiple lung nodules.

Results of the tumor cell phenotypes are shown in Table 4.

In the graft recipients, tumor Ig expression was monoclonal for IgMk in three cases (patients 3, 6, and 7), monoclonal Igk without clear demonstration of heavy chain isotype in 1 case (patient 4) and polyclonal with no light chain restriction in 2 cases (patients 5 and 8). In two cases (patients 1 and 2), the absence of detectable Ig on the tumor cells was interpreted as genuine since no staining could be achieved with either snap-frozen or conventionally processed tissue. Polyclonal Ig was detected in all tumor samples from both XLPS cases.

All samples were tested for the presence of EBNA proteins. Positive staining for EBNA-1 and EBNAs 1–4 and 6 was clearly demonstrated in all samples with immune human sera JAT and RT. Although qualitatively, the staining intensity was greater with the serum containing activity to all EBNA species, except EBNA-5, there were no apparent differences in the cellular distribution of the two reagents. Most tumor cells exhibited intense granular nuclear activity (Fig. 2a) that emphasized their nuclear pleomorphism. Smaller numbers of cells showed less intense staining, often limited to a few labeled nuclear granules. This heterogeneous staining pattern was more typical of the pattern observed in the B958 B-LCL–positive control (Fig. 2b) than the homogeneous distribution of EBNA in the Raji BL control. A smaller proportion of morphologically similar cells were EBNA-2 (Fig. 2c) and EBNA-3-positive in tissues from 9 and 5 cases, respectively. Apart from the biopsy from patient 4, which contained a high proportion (≈50%) of EBNA-2–positive cells, positive staining was observed on <5% of the tumor cell population in these samples. Similarly, LMP was present on a minority population in 5 patient samples. This was more frequently detected with mAb pool CS1-4 than mAb S12, although both reagents were reactive with the control B958 BLCL. None of the lesions expressed VCA or MA, and no EA was demonstrated in 4 samples tested. Reliable detection of the mAbs to EBNA proteins was consistently achieved with immunoperoxidase and fluorescence methods, whereas all labels, including APAAP, proved suitable for labeling of lytic cycle proteins on control cells and LMP.

The extent of the phenotyping studies was limited by the amount of biopsy tissue available but in all cases the B cell lineage of the tumor could be confirmed by the presence of CD45 antigen, together with the broadly reactive B cell markers CD22 and L26 (Fig. 3a) and absence of cytokeratin activity. However in the transplanted patients, the pan–B cell marker CD19 associated with most B cell differentiation stages was absent except in 2 samples (patients 1 and 6). Similarly, CD21 antigens were detected in only 2 cases (patients 1 and 3) and CD38 antigen in a proportion (<50%) of tumor cells from patient 4. Although tumors from both XLPS patients failed to express most B cell differentiation antigens except CD19 in patient 10, the presence of CD38 antigens in these samples confirmed the plasmacytoid nature of the lesions in contrast to the majority of those arising in the organ transplant recipients. A more distinct pattern between the two groups of patients emerged with respect to the CAM expression. Whereas moderate or weak LFA-1, LFA-3, and ICAM-1 staining was detected on the majority of cells in lesions from six transplant recipients, LFA-3 activity was present in one XLPS (patient 10) only. None of the tumors in five samples tested expressed BLA. All samples tested from both patient groups expressed monomorphic class I and class II (HLA-DR) determinants (data not shown) although weak class II antigen staining of the tumor populations in 4 samples (patients 2, 4, 5, and 7) contrasted with the intense reaction seen on accompanying reactive cells. Class II sublocus products HLA-DP and HLA-DQ were also expressed, albeit weakly, in four of five and three of four samples tested but were not detectable in two cases (patients 2 and 8).

FIGURE 3. (a) Paraffin section of posttransplant B cell lymphoma (patient 8) stained with pan-B cell marker, mAb L26, using PAP method (×630). (b) Cryostat section of posttransplant B cell lymphoma (patient 4) shows CD3-positive T cells infiltrating (unstained) tumor cell population demonstrated with mAb T3 and the PAP method (×630).

Extended studies of the accompanying normal or reactive cell populations were performed on samples from 4 transplant (patients 3, 4, 5, and 8) and both XLPS patients. Small lymphoid cells appeared to be almost exclusively mature CD2-, CD3-positive T cells that coexpressed class II (HLA-DR) antigens and contained various numbers of the CD4 and CD8 T cell subsets. Substantial numbers of both CD4- and CD8-positive T cells were present within the tumor populations of the nodal lesions from patients 5 and 8 and, although there were fewer, in the abdominal tumor from patient 4. These cells exhibited a distinct dendritic morphology (Fig. 3b). By contrast, sparse and mainly CD8 positive T cells were detected in the autopsy samples from patient 3 and both XLPS cases, all of whom had widespread disease.

Tissue typing studies in two of the organ transplant recipients who had received HLA-incompatible grafts showed in each case that the tumors were of host/recipient origin. In patient 1 who was HLA-A3$^+$, DR3$^-$ and was grafted with renal tissue

from an HLA-A3$^-$, DR3$^+$ donor, HLA-DR3 antigen staining was demonstrated on residual normal structures of the grafted organ but was absent on the adjacent tumor. Conversely, mAbs to HLA-A3 showed positive staining of the tumor cells only. In the second case (patient 6) who was HLA-A3$^-$ and had received a heart/lung graft from an HLA-A3$^+$ donor, absence of staining on the tumor implied host origin, whereas HLA-A3$^+$ vascular or lung alveolar structures were presumed to be donor-derived.

Western immunoblotting studies. In confirmation of the immunocytochemical demonstration of different EBNA components, immunoblots of tissue extracts from patient 2 showed bands corresponding to EBNA-1 and EBNA-2 with serum RT. Faint bands consistent with high-MW EBNAs-3,4,6 were additionally demonstrated (Fig. 4a). A similar immunoblot probed with anti-LMP mAbs CS1-4 also revealed LMP expression in case 2 (Fig. 4b). This intense LMP reaction contrasted with the relatively weak staining of low cell numbers in tissue sections of the same sample using mAbs CS1-4, suggesting that the expression of some of these proteins may be below the level of detection by immunocytochemical methods.

DISCUSSION

The most important finding of this study was that the pattern of EB virus gene expression in B cell tumors from eight organ transplant recipients and two XLPS patients suggested a latent/nonproductive type of infection. All lesions from the transplant group and one XLPS case expressed EBNA-1 and EBNA-2 with additional EBNA-3 and/or LMP in eight cases. EBNA-1 and LMP alone were detected in the remaining XLPS case. The presence of these latent gene products was routinely demonstrated on cryostat sections using immunocytochemical techniques and in one case (patient 2) by immunoblotting, which additionally demonstrated high-molecular-weight bands consistent with EBNAs 3, 4, and 6. No lytic cycle antigens were detected in any of the tissues studied. The presence of EBNA components with LMP typifies the gene expression in EB virus–induced immortalization in vitro. This pattern of viral gene expression in marked contrast to the exclusive expression of EBNA-1 seen in BL shown by immunoblotting with tumor extracts (*33*). The contrasting patterns of viral gene expression may reflect the origins of the two types of tumor. Since BL arises in a seropositive host with relatively normal immunoregulatory controls for EB virus (*34*), T cell surveillance would be expected to remove infected cells expressing viral antigens, such as EBNA-2 and LMP (*35*, *36*), that have been shown to be targets of T cell cytotoxicity. Under these conditions, a tumor can only arise if, due to as yet undefined factors, an infected cell had extremely restricted viral gene expression. Conversely, in transplant recipients, immunosuppressive therapy has been shown to inhibit EB virus–specific cytotoxic T cells allowing latently infected B cells to survive in vivo (*37*). However, since EB virus–associated B cell tumors arise in only a small percentage of transplant recipients (*38*), other immune mechanisms must be effective in controlling the proliferation of these cells. It is interesting that the pattern of viral gene expression seen in this series of postgrafting tumors is identical to that reported in tumors arising after experimental EB virus infection in cotton-top tamarins (*39*). The immune response of seronegative animals to a large dose of parentally administered virus appears to be unable to control the EB virus–driven B cell proliferation that clinically mimics that seen in the transplant recipients. The viral gene expression in both the human and experimentally induced animal tumors is similar or identical to that which has been shown to cause immortalization in vitro. It is therefore tempting to speculate that EB virus is the sole agent involved in the pathogenesis of these lesions. In contrast, the development of BL is thought to be multifactorial

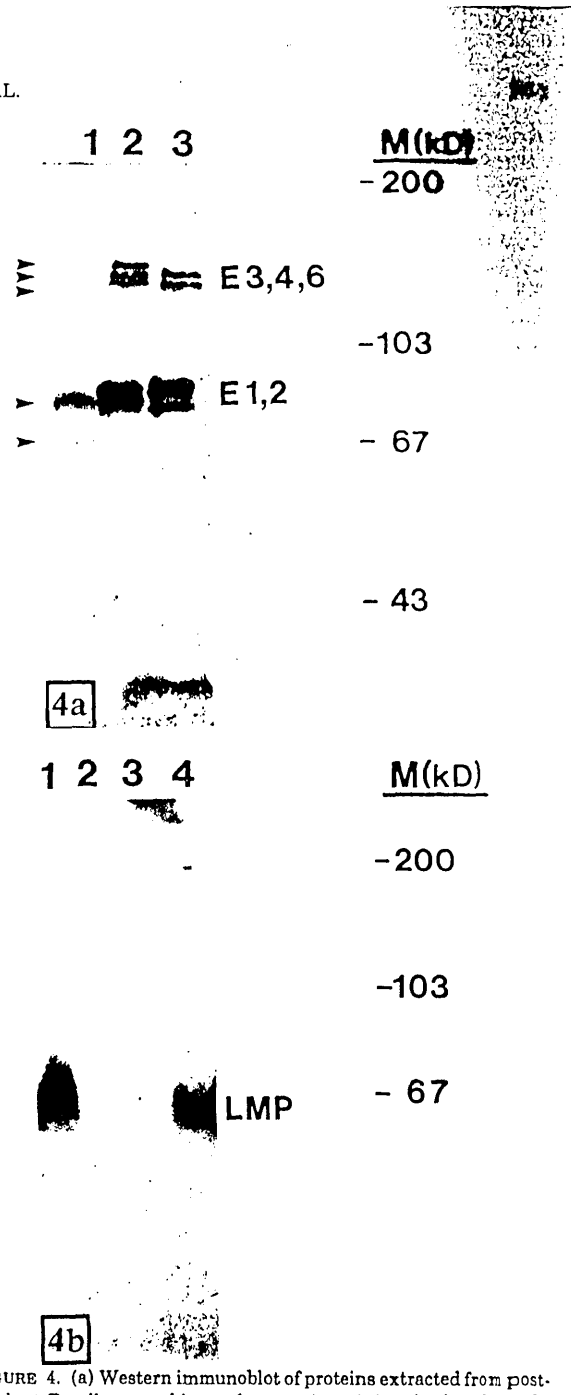

FIGURE 4. (a) Western immunoblot of proteins extracted from post-transplant B cell tumor biopsy from patient 2 (track 1); LCL, 2C9 (track 2); and LCL 1134 (track 3) and probed with human serum RT. EBNA species 1,2,3,4, and 6 in the tumor are indicated (arrow). (b) Western immunoblot of proteins extracted from patient 2 tumor tissue (track 4); LMP-positive BL cell line, P3HR1 (track 1); LMP-negative BL cell lines AW Ramos (track 2); and BL41 (track 3), and probed with anti-LMP mAbs CS1-4, shows a band corresponding to LMP in the tumor tissue extract.

with EB virus as one of a number of essential pathogenetic cofactors.

The preponderance of recent primary infections in the patients of this study has also been reported by others (*14*). While some individuals developing primary infection during immunosuppressive therapy may be at risk of developing tumors, this has not been consistently reported in all transplant series (*30*). The atypical low antibody responses to primary infection demonstrated in these patients (*30*) may in fact be influenced by postgrafting blood transfusions as well as immunosuppression. Even when associated with a primary infection, some of the postgrafting tumors in this study differed from those arising in the XLPS patients with respect to monoclonal Ig expression. This suggests an additional event necessary for the outgrowth of malignant cells. In the XLPS patients, the polyclonal proliferation resulted in widespread disease that may reflect the specific immune deficit in these cases.

The pattern of cellular gene expression in the lesions in the transplant recipients was reminiscent of lymphoblastoid (*20*) rather than BL cells (*20*) in which the expression of B cell activation markers and cell adhesion molecules is low or undetectable (*18*). This finding is not surprising since recent transfection experiments have shown that LMP expression causes upregulation of CAMs (*40*) and EBNA-2 expression causes upregulation of CD23 (*41*). Absence of CAMs on BL cells would contribute to their ability to evade the immune response and, similarly, the absence of LFA-1 and ICAM-1 in the XLPS lesions may be significant factors in the rapid dissemination of these tumors. The selective expression of CD23 antigen in the two groups of patients also suggests specific stages of B cell differentiation within the tumors. CD23 antigens are present on activated normal B cells and normal B cells immortalized by EB virus in vitro but absent on immature pre-B cells and terminally differentiated plasma cells (*5*, *42*). The apparent restriction of CD23 antigen expression in the postgrafting tumors suggests that these lesions may represent an early stage of EB virus induced B cell activation. The absence of CD23 antigens in the XLPS samples might indicate an immature, pre-B cell proliferation, but it is more likely that these cells correspond to a more advanced stage of B cell activation given the predominantly plasmacytoid nature of the lesions and their cellular expression of the CD38 plasma cell associated antigen. In line with this, the failure to detect CD21 EB virus receptor antigens in most cases is consistent with evidence that these are lost from resting B cells soon after EB virus-induced activation (*5*).

The recipient origin of the tumor cells suggested by tissue typing studies in two postgrafting tumors contrasts with findings in allogeneic bone marrow transplant recipients in which the B cell proliferations are usually donor-derived (*43*). In one case of this study, the recipient origin could only be inferred by the absence of relevant polymorphic class I determinants since adequate tissue was not available for extensive typing with monomorphic reagents, and recent reports have shown that HLA-ABC antigens may be selectively lost in some malignancies (*44*). Since some of the lesions developed within grafted organs it is possible that the immunological microenvironments of these tissues provide a growth advantage to EB virus-infected host cells, although it is not known whether occult tumor existed at other sites.

The morphological appearances of B cell lymphoma in the transplant recipients was supported by monoclonal Ig expression in 3 cases. Absence of light-chain restriction in 2 cases is not an uncommon feature of these tumors (*16*, *45*) and does not exclude a lymphomatous process since Ig light chains may not be demonstrable by immunocytochemistry in some immunoblastic or lymphoblastic lymphomas (*46*). However, the existence of clonal Ig gene rearrangements in these samples and in tumors with polyclonal Ig expression cannot be excluded since the use of highly sensitive molecular genetic techniques has now shown that the majority of posttransplant lymphomas contain monoclonal B cell populations (*45*).

These studies also showed that the nonneoplastic component in some samples contained phenotypically mature T cells with varying numbers of both CD4 and CD8 T cell subsets. This observation contrasts with the unusual prevalence of immature T cells lacking CD4 and CD8 antigens reported in the peripheral blood of long-term renal allograft recipients (*47*). The high T cell numbers in three posttransplant tumor biopsies may have been influenced by the location of the tumors in lymphoid-associated tissues. It is salient, however, that the lesions in these cases also showed a rapid response to therapy, providing further support to the importance of our findings of both CAM and EB virus latent gene expression in tumors arising after transplantation. The reversibility of these lesions after withdrawal of immunosuppression suggests that once normal T cell responses are restored, cellular interactions can proceed unimpeded, allowing tumor regression. By contrast, the rapidly progressive tumors in three patients, including both XLPS patients, contained few T cells—which, together with the failure to consistently express latent gene products and cytoadhesion molecules, may be significant contributory factors in allowing these tumors to evade immunosurveillance.

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