EPSTEIN-BARR VIRUS

1. Virus–Host Interactions

1.1 Structure, taxonomy and viral gene products

1.1.1 *Structure*

Like other herpesviruses (see Figure 1 of Introduction), Epstein-Barr virus (EBV) has a toroid-shaped protein core that is wrapped with DNA, a nucleocapsid with 162 capsomers, a protein tegument between the nucleocapsid and the envelope and an outer envelope with external glycoprotein spikes. The major EBV capsid proteins are 160, 47 and 28 kDa, similar in size to the major capsid proteins of herpes simplex virus. The most abundant EBV envelope and tegument proteins are 350/220 and 152 kDa, respectively (Kieff, 1996).

1.1.2 *Taxonomy*

EBV is the most extensively studied gammaherpesvirus and the prototype of the lymphocryptovirus subfamily. The gammaherpesvirus classification was initially established on the basis of similarity in biological properties rather than structural characteristics. Like other gammaherpesviruses, EBV establishes latent infection in lymphocytes and can induce proliferation of the latently infected cells. Taxonomists have renamed EBV human herpesvirus 4 (HHV4).

Two major EBV types have been detected in humans (Gerber *et al.*, 1976a; Adldinger *et al.*, 1985; Zimber *et al.*, 1986; Young *et al.*, 1987; Rowe *et al.*, 1989; Sixbey *et al.*, 1989; Gratama *et al.*, 1992; Walling *et al.*, 1992). Type 1 and type 2 EBV genomes (formerly referred to as types A and B) are nearly identical, except for the genes that encode some of the nuclear proteins (EBV nuclear antigen, EBNA-2, EBNA-3A, -3B, -3C; see section 1.5.2) in latently infected cells (Nonoyama & Pagano, 1973; Bornkamm *et al.*, 1980). These differences are reflected in type-specific and type-common epitopes for antibody (Young *et al.*, 1987) and T-cell responses (Moss *et al.*, 1988). Antibodies to type-2 viruses are somewhat more prevalent in African sera than in sera from people in developed countries, but the recovery of type-2 virus from blood is unusual (Young *et al.*, 1987; Rowe *et al.*, 1989), perhaps because EBV type-2 infected lymphocytes grow less efficiently *in vitro* (Rickinson *et al.*, 1987). A naturally occurring EBV recombinant that encodes both type-1 and type-2 nuclear antigen sequences was isolated from the blood of a healthy adult in Papua–New Guinea (Burrows *et al.*, 1996a).

In addition to type-specific polymorphism, significant DNA sequence heterogeneity has been found by comparison of selected regions of the genome in EBV isolated in certain geographical areas or even from the same area. These polymorphisms define

different viral strains within types 1 and 2 (Aitken *et al.*, 1994). Some of the changes cause amino-acid substitutions in viral proteins and may even affect peptides that are important for the immune control of viral infection (de Campos-Lima *et al.*, 1993a; Lee *et al.*, 1993a; de Campos-Lima *et al.*, 1994; Lee *et al.*, 1995a; Burrows *et al.*, 1996b).

1.1.3 *Host range*

Under normal circumstances, EBV infection is restricted to humans. Antibodies to EBV have been detected in several primate species, probably due to the presence of cross-reactive antibodies against their own species-specific EBV homologues (reviewed by Kieff *et al.*, 1979). Such cross-reactive immunity may explain why these animals are usually refractory to experimental infection, although their B lymphocytes can be efficiently transformed *in vitro* (Miller *et al.*, 1972; Levine *et al.*, 1980a). New World monkeys carry no EBV-like virus, and at least three species appear to be infectable experimentally. The cotton-topped tamarin (*Saguinus oedipus oedipus*) and owl monkey (*Aotus trivirgatus*) are susceptible to EBV-induced B-cell lymphomas (Epstein *et al.*, 1973; Shope & Miller, 1975), while in more limited studies with the common marmoset (*Callithrix jacchus*) induction of a mononucleosis-like syndrome was seen in some animals (Wedderburn *et al.*, 1984). Infection of newborn marmosets with EBV resulted in the establishment of a long-term permissive infection, indicating similarities in the responses of marmosets and humans to EBV (Cox *et al.*, 1996).

1.1.4 *Target cells*

EBV is a B-lymphotropic herpesvirus, and infection of B cells is mediated through interaction of the viral envelope glycoprotein gp350 (gp340)/220 with the receptor for the C3d complement component CR2 (CD21; Fingeroth *et al.*, 1984, 1988). After binding of the viral particle to the surface of the host cell and endocytosis, the viral envelope fuses with the host cell membrane by a mechanism involving three other viral glycoproteins, gp85, gp25 and gp42 (Li *et al.*, 1995a). Interestingly, gp42 can bind to major histocompatibility complex (MHC) class II, and EBV uses this as a cofactor in the infection of B lymphocytes (Li *et al.*, 1997). The notion that EBV is a predominantly if not exclusively B-lymphotropic virus has been challenged, initially on the basis of the detection of EBV in non-B-cell tumours, notably certain T-cell lymphomas and some carcinomas. In addition, it has become apparent that expression of the C3d/EBV receptor is not restricted to B cells. Thus, identical or related molecules have been detected in T cells, follicular dendritic reticulum cells and, possibly, some epithelial cells (Reynes *et al.*, 1985; Fingeroth *et al.*, 1988; Timens *et al.*, 1991; Birkenbach *et al.*, 1992; Hedrick *et al.*, 1992; Sinha *et al.*, 1993). The cellular tropism of EBV is clearly not restricted through CD21, however; thus, the virus has been detected in CD21-negative cells. Moreover, some CD21-positive cells, such as follicular dendritic reticulum cells, have proved difficult to infect *in vitro* and have been shown consistently to contain no EBV *in vivo*.

Current evidence suggests that EBV infection in healthy chronic virus carriers is largely restricted to B cells, although in certain situations the virus can be detected in non-neoplastic epithelial cells and T lymphocytes as well (see section 1.2.1).

1.1.5 *Genome, episomal and integrated viral forms and gene products*

The EBV genome is a linear, double-stranded, 172-kb DNA molecule (Kieff, 1996). It has been speculated that the observed relative underrepresentation of CpG dinucleotides may correlate with the latent status of the viral genome (Honess *et al.*, 1989). The genome of the prototype laboratory strain B95-8 has been sequenced as a set of *Bam*HI-restriction fragments (Baer *et al.*, 1984). The nomenclature for EBV open reading frames (ORFs) is based on the *Bam*HI-restriction fragment in which they are found. For example, the *BARF1* ORF is found in the *Bam*HI A fragment and extends rightwards. The *BLLF2* ORF is the second leftwards ORF in the *Bam*HI L fragment (Table 1, Figure 1). The genome has the structural organization of group C herpesviruses with reiterated 0.5-kb terminal direct repeats and a reiterated 3-kb internal direct repeat, which divide the genome into short and long, largely unique sequences. The unique sequences contain several perfect or imperfect tandem repeat elements, many of which encode for repeat domains in proteins. EBV isolates differ in their tandem repeat reiteration frequency. Thus, differences in protein size are often useful markers of specific isolates and strains (Gratama *et al.*, 1990). Furthermore, latent EBV infection is usually characterized by persistence of the genome as an episome with a characteristic number of terminal repeats. The fact that each progeny cell tends to have the same number of terminal repeats can be used to determine whether latently infected cells have arisen from a common progenitor (Raab-Traub & Flynn, 1986).

Circular episomal EBV DNA is detected in the nuclei of blood lymphocytes infected *in vitro* within 12–16 h of infection (Hurley & Thorley-Lawson, 1988). Probably only DNA repair synthesis is required for circularization, because significant nucleotide incorporation does not occur until 48 h after infection (Sixbey & Pagano, 1985). Stable, latently infected, proliferating so-called 'lymphoblasts' contain multiple EBV episomes (Adams & Lindahl, 1975; Kaschka-Dierich *et al.*, 1976; Lindahl *et al.*, 1976). EBV episomes are replicated early in the S phase by cell DNA polymerase (Hampar *et al.*, 1974; Gussander & Adams, 1984; Shaw, 1985); the initial amplification requires DNA synthesis early in the S phase (Hampar *et al.*, 1974), and the number of episomes increases thereafter over several days (Sugden *et al.*, 1979), probably due to effects of EBNA-1 on S-phase *oriP*-dependent DNA replication, although the abundance of EBNA-1 does not appear to correlate with the episome copy number (Ernberg *et al.*, 1977; Yates *et al.*, 1984; Sternås *et al.*, 1986). Episomal DNA is likely to be necessary for lytic cycle EBV DNA replication, since viral production has not been observed in cells that contain only integrated EBV DNA, and circular DNA copy numbers increase during lytic infection (Shaw, 1985).

Although episomal forms are commonest, the EBV genome can also persist by integrating into chromosomal DNA (Henderson *et al.*, 1983; Matsuo *et al.*, 1984; Lawrence *et al.*, 1988; Hurley *et al.*, 1991a,b; Delecluse *et al.*, 1993a). Few cell lines with integrated EBV DNA have been studied in detail. In the Burkitt's lymphoma line Namalwa, EBV DNA has persisted by integrating into 1p35, and the entire block of cell DNA with the integrated EBV genome is duplicated as an inverted DNA domain

Table 1. Identified EBV gene products and their open reading frames

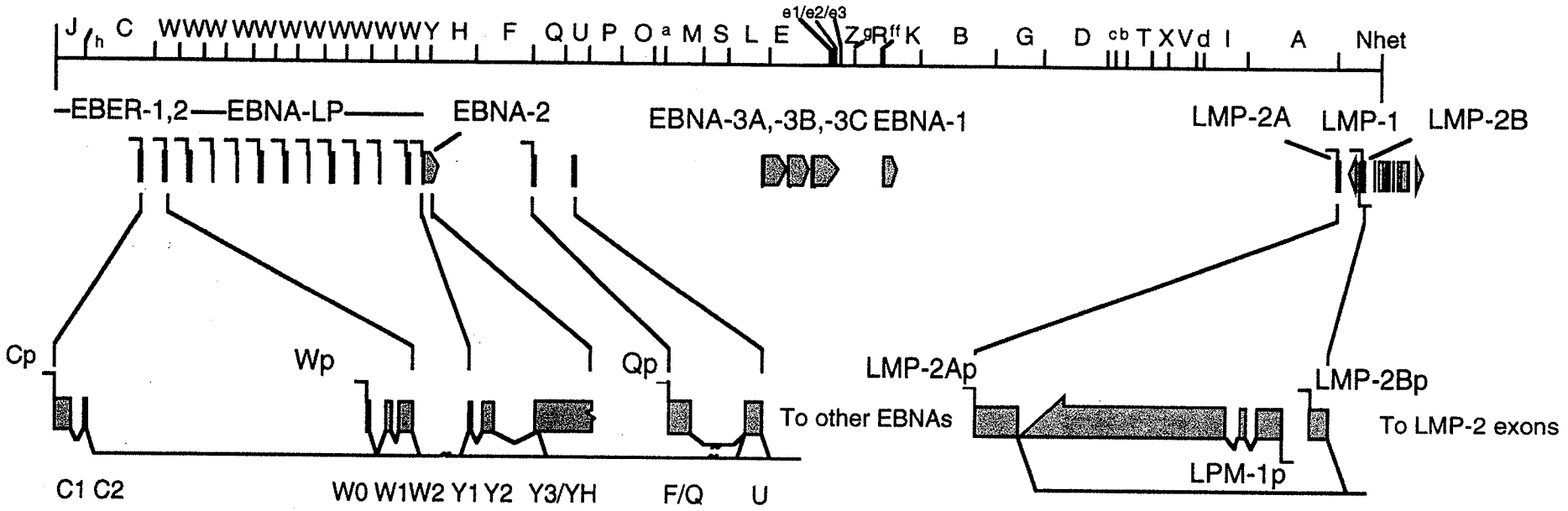
Open reading frame	Common name	Proposed function
Latent genes		
BKRF1	*EBNA-1*	Plasmid maintenance
BYRF1	*EBNA-2*	*trans*-Activation, transformation
BERF1	*EBNA-3A*	*trans*-Activation, transformation
BERF2	*EBNA-3B*	Unknown
BERF3/4	*EBNA-3C*	*trans*-Activation, transformation
BWRF1	*EBNA-LP*	*trans*-Activation, transformation
BNLF1	*LMP-1*	Transformation
BNRF1	*LMP-2A/2B*	Maintenance of latency
BARF0[a]		Unknown
Immediate early genes		
BZLF1	*ZEBRA*	*trans*-Activation, initiation of lytic cycle
BRLF1		*trans*-Activation, initiation of lytic cycle
BI'LF4		*trans*-Activation, initiation of lytic cycle
Early genes		
BMRF1		*trans*-Activation
BALF2		DNA binding
BALF5		DNA polymerase
BORF2		Ribonucleotide reductase subunit
BARF1		Ribonucleotide reductase subunit
BXLF1		Thymidine kinase
BGLF5		Alkaline exonuclease
BSLF1		Primase
BBLF4		Helicase
BKRF3		Uracil DNA glycosylase
Late genes		
BLLF1	*gp350*	Major envelope glycoprotein
BXLF2	*gp85 (gH)*	Virus–host envelope fusion
BKRF2	*gp25 (gL)*	Virus–host envelope fusion
BZLF2	*gp42*	Virus–host envelope fusion, binds MHC class II
BALF4	*gp110 (gB)*	Unknown
BDLF3	*gp100–150*	Unknown
BILF2	*gp55–80*	Unknown
BCRF1		Viral interleukin-10
BHRF1[a]		Viral *bcl-2* analogue

Adapted from Li *et al.* (1995a); Nolan & Morgan (1995)

[a] Expressed in latently infected cells as well

EBNA, EBV nuclear antigen; LP, leader protein; LMP, latent membrane protein; ZEBRA, Z EBV replication activation; gp, glycoprotein; MHC, major histocompatibility complex

Figure 1. *Bam*HI-restriction map of the EBV genome and latent transcripts



Adapted from Masucci and Ernberg (1996) and Rickinson and Kieff (1996)
EBER, EBV-encoded RNA; EBNA, Epstein-Barr nuclear antigen; LMP, latent membrane protein
Other abbreviations are explained in the text.

(Henderson *et al.*, 1983; Lawrence *et al.*, 1988). In the human fetal lymphoblastoid cell line IB4, multiple EBV genome copies are integrated in tandem into 4q25 (Matsuo *et al.*, 1984; Hurley *et al.*, 1991b). Integration occurs frequently when Burkitt's lymphoma cells are infected with EBV *in vitro* (Hurley *et al.*, 1991a). Clearly, integration is not chromosome site-specific or a regular feature of EBV infection.

In the establishment of latent infection, most of the EBV genome undergoes progressive methylation (Kintner & Sugden, 1981; Larocca & Clough, 1982; Perlmann *et al.*, 1982), whereas regulatory domains involved in maintaining latent infection, such as *oriP*, tend to remain under-methylated (Minarovits *et al.*, 1992). Extensive methylation of parts of the genome not expressed in latent infection may help to maintain latency by inhibiting lytic gene expression (Nonkwelo & Long, 1993). Treatment of latently infected cells with drugs that reduce DNA methylation increases the frequency of cells entering the productive cycle (Ben-Sasson & Klein, 1981).

EBV infection of primate B lymphocytes *in vitro* is usually nonproductive and results in the expression of a restricted number of viral genes and continuous proliferation. EBV-infected proliferating B lymphocytes are similar to lymphocytes that are proliferating in response to antigens, mitogens or interleukin (IL)-4 and anti-CD40 in that they express a similar repertoire of activation-associated markers, secrete similar immunoglobulins and have similar intercellular adherence (Åman *et al.*, 1986; Hurley & Thorley-Lawson, 1988; Alfieri *et al.*, 1991; Banchereau *et al.*, 1991). At least 11 EBV genes are expressed in latently infected B lymphocytes (Table 2, Figure 1). Two of these encode small, non-polyadenylated RNAs (the EBV-encoded RNAs EBER-1 and EBER-2), six encode nuclear proteins (the EBV nuclear antigens EBNA-1, EBNA-2, EBNA-3, -4 and -6, also called EBNA-3A, -3B and -3C, and EBNA-5, also called leader protein, LP), and three encode integral membrane proteins (the latent membrane proteins LMP-1 and LMP-2A and -2B, also called terminal proteins 1 and 2). Other viral mRNAs encoded for example by the *BHRF1* and *BARF0* ORFs have been detected in latently infected cells, but their products are not well characterized (reviewed by Kieff, 1996).

Different patterns of viral gene expression have been identified in latently infected, EBV-carrying cells *in vivo* (Table 3). Expression of all the latent viral genes detected in lymphoblastoid cell lines *in vitro* is a feature of the immunoblastic lymphomas of immunosuppressed patients. This form of latency is commonly defined as latency III. Latency I was first detected in EBV-positive Burkitt's lymphoma cells and is characterized by restricted expression of the EBERs, EBNA-1 and probably the product of the *BARF0* ORF. Latency II, characterized by the expression of EBERs, EBNA-1 and the three LMPs, was first recognized in a proportion of nasopharyngeal carcinomas but also seems to be common in most EBV-carrying non-B-cell tumours and in Hodgkin's disease (reviewed by Rickinson & Kieff, 1996). The latency II type has been reproduced *in vitro* by stable infection of a human T-cell line with a recombinant EBV carrying a selectable marker (Yoshiyama *et al.*, 1995). An additional form of latency characterized by restricted expression of EBERs, EBNA-1 and EBNA-2 has been described in smooth-muscle tumours arising in immunosuppressed children and AIDS patients (Lee *et al.*, 1995b).

Table 2. Nomenclature of latent viral gene products

Adopted terminology	Alternative nomenclature	
EBNA-1	EBNA-1	EBNA-1
EBNA-2	EBNA-2	EBNA-2
EBNA-3A	EBNA-3	EBNA-3A
EBNA-3B	EBNA-4	EBNA-3B
EBNA-3C	EBNA-6	EBNA-3C
EBNA-LP	EBNA-5	EBNA-4
LMP-1		
LMP-2A	TP-1	
LMP-2B	TP-2	
EBER-1		
EBER-2		

EBNA, EBV nuclear antigen; LP, leader protein; LMP, latent membrane protein; EBER, EBV-encoded RNA; TP, terminal protein

Table 3. Patterns of EBV latent gene expression

Type of latency	Gene product	Examples	Reference
I	EBERs, EBNA-1	Burkitt's lymphoma	Rowe *et al.* (1987a)
		Gastric carcinoma	Imai *et al.* (1994a)
II	EBERs, EBNA-1, LMP-1, -2A, -2B, BARF0	Hodgkin's disease	Deacon *et al.* (1993)
		Nasopharyngeal carcinoma	Hitt *et al.* (1989); Brooks *et al.* (1992)
III	EBERs, EBNA-1, -2, -3A, -3B, -3C, -LP LMP-1, -2A, -2B	Post-transplant lympho-proliferative disorder	Young *et al.* (1989a)
		Infectious mononucleosis	Tierney *et al.* (1994)
Other	EBERs, EBNA-1, -2	Smooth-muscle tumours	Lee *et al.* (1995b)

EBER, EBV-encoded RNA; EBNA, EBV nuclear antigen; LMP, latent membrane protein; LP, leader protein

With a frequency characteristic of each EBV-infected B-lymphocyte line, some progeny spontaneously become permissive for viral replication. In these cells, the viral DNA is amplified several hundred-fold by a lytic origin of DNA replication, *oriLyt* (Hammerschmidt & Sugden, 1988). Multimeric forms produced from presumably circular templates are generated from *oriLyt* following a pattern that could result from rolling-circle DNA replication (Pfüller & Hammerschmidt, 1996). The expression of viral genes associated with productive infection follows a temporal and sequential order (reviewed by Kieff, 1996). Some viral genes are expressed early after induction, indepen-

dently of new protein synthesis, and are therefore classified as immediate early genes. Early lytic genes are expressed slightly later, and their expression is not affected by inhibition of viral DNA synthesis. Genes the expression of which is blocked by inhibitors of viral DNA synthesis are categorized as late genes. Because epithelial cells are fully permissive for lytic EBV infection *in vivo* (Lau *et al.*, 1993; Ryon *et al.*, 1993), considerable efforts have been made to adapt EBV to grow in organ cultures of transformed epithelial cell lines. These cultures can be infected, but the infection is often abortive or inefficient and very little virus is produced (Li *et al.*, 1992). Therefore, most of our knowledge of latent or lytic EBV products is derived from the study of EBV-infected B lymphocytes.

1.1.5.1 *EBV-encoded RNAs*

The two EBV-encoded, small, non-polyadenylated RNAs (EBER-1 and -2) are by far the commonest viral RNAs in latently infected cells, with an estimated abundance of 10^7 copies per cell (Howe & Steitz, 1986; Howe & Shu, 1989). They are usually transcribed by RNA polymerase III, although polymerase II may also be involved (Howe & Shu, 1989). Most EBERs are located in the nucleus and are associated at the 3′ terminus with the cellular La antigen (Howe & Steitz, 1986) and other cellular proteins, including the EBER-associated protein, which shows strong resemblance to an endodermal, developmentally regulated sea-urchin protein called 217 (Toczyski & Steitz, 1991). The primary sequences of EBER-1 and -2 show extensive similarity to those of adenovirus VAI and VAII and cell U6 small RNAs (Glickman *et al.*, 1988). These RNAs form stable secondary structures that allow extensive intermolecular base-pairing and may have, therefore, similar functions. On the basis of the known function of the RNAs of VA and U6, two alternative roles have been proposed for the EBERs. In adenovirus infection, VAI RNA inhibits the activation of an interferon-induced protein kinase which blocks transcription by phosphorylating the protein-synthesis initiator factor eIF-2 α. EBER-1 and -2 can partially complement the replication of an adenovirus with null mutations in VAI and VAII, but their effect on eIF-2 α kinase activity is significantly smaller and they are not found in the cytoplasm (Kieff, 1996). A possible role of the EBERs in the splicing of the primary EBNA and LMP mRNA transcripts is suggested by the partial complementarity to RNA splice sites. Complexes between U6 and U4 RNAs are required for RNA splicing *in vitro*, and the EBER-2 RNA contains a single-stranded loop with six of seven nucleotides identical to those required for U6–U4 interaction (Glickman *et al.*, 1988). Both of the proposed functions of EBERs are somewhat incompatible with the observation that their expression is delayed until after EBNA and LMP gene expression and initiation of DNA synthesis (Alfieri *et al.*, 1991). Nevertheless, the earlier events in primary B-cell infection are sensitive to interferon (IF) (Thorley-Lawson, 1980, 1981), and EBERs may play a role in blocking eIF-2 kinase. EBV recombinants in which the EBERs have been deleted can initiate primary B-cell infection and growth transformation in the same way as wild-type virus, and no differences were seen in the growth of lymphoblastoid cell lines infected with EBER-deleted and control virus or in the permissiveness of these cells for lytic infection (Swaminthatan *et al.*, 1991). The EBER

transcription unit was found to be unmethylated in all types of EBV-carrying cells (Minarovits *et al.*, 1992).

1.1.5.2 *Nuclear proteins*

Transcription of nuclear proteins is initiated at polymerase II-dependent promoters in the *Bam*HI C (Cp) and *Bam*HI W (Wp) regions (reviewed by Rogers *et al.*, 1992). Approximately at the time of genome circularization, the Wp promoter initiates rightwards transcription that proceeds through almost the entire strand of the genome (Bodescot *et al.*, 1986). The mRNAs contain a 40-base first exon, W0 (Rogers *et al.*, 1992), which is spliced to alternating W1 and W2 exons derived from successive reiteration of the internal *Bam*HI W repeats (Bodescot & Perricaudet, 1986; Sample *et al.*, 1986). The use of an alternative splice acceptor site in W1 results in generation of an ATG codon in about half of the transcript. This initiates translation of an ORF encoded by the repeating W1 and W2 exons and by two short exons in the *Bam*HI Y region (Y1 and Y2). The resulting protein, called EBNA-LP, is encoded in the leader of the EBNA mRNAs and is therefore also called leader protein (LP) (Kieff, 1996).

The Y2 exon is spliced to a long exon, YH, that spans the right end of *Bam*HI W and the left end of *Bam*HI H (Kieff, 1996). The ORF of this exon encodes the nuclear protein EBNA-2. EBNA-LP and EBNA-2 are the first viral proteins expressed in infected B lymphocytes. They are already detected 12 h after infection and reach the levels maintained in transformed lymphoblastoid cell lines within 24–32 h after infection (Alfieri *et al.*, 1991). Probably as a consequence of the action of EBNA-2 on response elements located in the upstream *Bam*HI C fragment, a switch from Wp to Cp usage occurs in most of the infected cells (Woisetschlaeger *et al.*, 1990). Two exons downstream of Cp, C1 and C2, replace the W0 exon in those transcripts. As with the W0 exon, alternative splicing of C2 to W1 or W0 results in mRNAs incapable or capable of translating EBNA-LP, respectively. A potential splice donor site near the beginning of the YH exon, but preceding the EBNA-2 initiation codon, is activated in some of the transcripts and is spliced to a downstream acceptor in *Bam*HI U. The U exon is further spliced to any of four alternate acceptor sites that begin the ORFs encoding EBNA-3A, EBNA-3B, EBNA-3C or EBNA-1. Thus, as a consequence of alternate 5′ splicing of Cp- or Wp-initiated RNAs, some RNAs will encode EBNA-LP or EBNA-LP and EBNA-2, while other RNAs will exclusively code for EBNA-3A, -3B, -3C or EBNA-1. The turning on of EBNA-3A, -3B, -3C and EBNA-1 expression is probably explained by the strength of the Cp, which leads to significantly more transcription. Polyadenylation may determine splicing and thereby regulate the expression of the six EBNA mRNAs from the same promoter. *cis*- or *trans*-Acting factors, such as the expression of certain viral products or the formation of secondary structures in the primary EBNA transcripts, may also play a role in splice choice.

In cells that express a latency I or II phenotype, the Cp and Wp are silenced and extensively methylated (Ernberg *et al.*, 1989; Schaefer *et al.*, 1997). Transcription of the EBNA-1 message is initiated at an alternative promoter located in the FQ transition region and, like the Cp- and Wp-initiated messages, splices to the U and K exons.

Characterization of the transcripts originating from this region has been complicated by the presence of several transcription–initiation start sites and by the overlapping of promoters that are active during the latent and lytic phases of the cycle. It seems now clear that the originally described Fp is a lytic promoter (Nonkwelo *et al.*, 1995, 1996), while transcription of the latency I EBNA-1 mRNAs begins at the adjacent Qp (Nonkwelo *et al.*, 1997).

(*a*) *EBNA-LP*

The size of EBNA-LP varies among isolates, owing to varying numbers of internal repeats (IR1) that contain the W1 and W2 exons of LP (Dillner *et al.*, 1986; Finke *et al.*, 1987; Wang *et al.*, 1987a). The W2 exon encodes for a 44-amino acid repeat containing basic Arg-Arg-His-Arg and Arg-Arg-Val-Arg-Arg-Arg domains that could be nuclear localization signals. EBNA-LP is phosphorylated, probably on serine residues located in the W2 exon near a casein kinase II phosphorylation site (Petti *et al.*, 1990; Kieff, 1996). The protein is strongly associated with the nuclear matrix and has an unusual location, as seen by immunofluorescence microscopy (Petti *et al.*, 1990; Jiang *et al.*, 1991). Some EBNA-LP is diffusely spread through the nucleus, while the rest is concentrated in a few granules, frequently distributed in curved linear arrays.

Molecular analysis of EBNA-LP has focused on the last two exons that are deleted in the nontransforming P3HR-1 EBV strain. Deletion of the exons resulted in EBV recombinants with significantly reduced growth transforming ability under standard in-vitro culture conditions (Allan *et al.*, 1992); however, only a modest reduction of transformation efficiency was observed by plating infected B lymphocytes in soft agarose over fibroblast feeder layers (Hammerschmidt & Sugden, 1989; Mannick *et al.*, 1991). The mutant virus-infected lymphoblastoid cell lines also tended to be more differentiated towards immunoglobulin secretion. The unusual fibroblast dependency and enhanced differentiation suggest that EBNA-LP may indirectly or directly up-regulate the expression of autocrine factors required for B-cell growth. Transient transfection of EBNA-LP and EBNA-2 into primary B lymphocytes co-stimulated with gp350 indicated that the two proteins cooperate in the induction of G0 to G1 transition, as marked by induction of cyclin D2 (Sinclair *et al.*, 1994), but the mechanism of this effect remains unknown.

(*b*) *EBNA-2*

EBNA-2 differs extensively between the EBV type-1 and type-2 isolates (Aitken *et al.*, 1994) and is the primary determinant of the biological differences that enable the type-1 strains to transform B lymphocytes with greater efficiency (Rickinson *et al.*, 1987). The two proteins consist of 484 and 443 amino acids, respectively; they are overall acidic, phosphorylated on threonine and serine residues and contain at least seven discrete domains including a polyproline repeat that varies by 10–40 amino acids in different EBV isolates. The carboxy-terminal domain contains two Arg-Gly and Lys-Arg-Pro-Arg repeat sequences that are required for nuclear localization (Cohen *et al.*, 1991). EBNA-2 localizes in large nuclear granules and is associated with nucleoplasmic, chromatin and nuclear matrix fractions (Petti *et al.*, 1990).

EBNA-2 is a specific *trans*-activator of cellular genes, such as the B-cell activation markers CD23 (Wang *et al.*, 1987b, 1990a, 1991) and CD21 (Wang *et al.*, 1990a) and the *c-fge* oncogene (Knutson, 1990), and viral genes including *LMP-1* (Abbot *et al.*, 1990; Ghosh & Kieff, 1990; Wang *et al.*, 1990b; Tsang *et al.*, 1991; Fåhraeus *et al.*, 1993) and *LMP-2* (Tsang *et al.*, 1991) and the *cis*-acting element upstream of the Cp (Sung *et al.*, 1991; Walls & Perricaudet, 1991; Sjöblom *et al.*, 1995a). The EBNA-2-responsive elements have been partially defined by deletion analysis of EBV promoter constructs or by positioning near a heterologous promoter (Laux *et al.*, 1994a). Molecular analysis of EBNA-2 by rescue of transforming virus from P3HR1-infected cells or the *trans*-activation of LMP-1 in transiently transfected Burkitt's lymphoma cells as phenotypic markers reveals a correlation between the sequences of EBNA-2 that are essential for transformation and those important for promoter *trans*-activating activity (Cohen *et al.*, 1991). Two large deletions, between amino acids 112–230 and 463–483, were shown to reduce transformation and *trans*-activating activity but still result in virus capable of transforming cells, albeit with low efficiency. Deletion of the Arg-Gly oligomer domain in residues 337–357 resulted in a 90% reduction of transforming efficiency but increased *trans*-activation of the LMP-1 promoter by at least 10-fold (Tong *et al.*, 1994; Kieff, 1996). This domain can interact with histones, potentially facilitating the interaction of EBNA-2 with DNA. Only three regions, located between residues 95–110, 280–337 and 425–462, appear to be stringently required for transformation and *trans*-activating activity. The 425–462 region is essential for its acidic *trans*-activating characteristics, while the 280–337 region mediates the interactions with DNA sequence-specific binding proteins that bring EBNA-2 to its responsive elements. The function of the 95–110 region is unclear (Kieff, 1996). Detailed analysis of the 425–462 region indicates that this domain is similar in many respects to the prototype VP16 acidic domain (Cohen & Kieff, 1991; Cohen, 1992), part of which can substitute for part of the EBNA-2 domain in the context of reconstituted EBV recombinants (Cohen, 1992). The two domains share affinity for the transcription factors TFIIB, TAF40, TFIIH and RPA70, suggesting a critical role of the 425–462 region in recruiting these factors to EBNA-2-responsive promoters (Tong *et al.*, 1995).

EBNA-2 does not interact directly with its responsive elements. The response elements in the LMP-1, LMP-2 and Cp promoters have been intensively investigated by electrophoretic mobility shift assays with nuclear extracts from EBNA-2-positive cells. Each responsive element contains at least two gel shift activities: the major activity corresponds to an oligonucleotide that includes the GTGGGAA motif (Zimber-Strobl *et al.*, 1991; Jin & Speck, 1992; Ling *et al.*, 1993a). A 28-amino acid polypeptide corresponding to residues 310–336 of EBNA-2 was used to affinity purify a 63-kDa nuclear protein that reproduced the gel shift pattern (Yalamanchili *et al.*, 1994). Sequencing of p63 showed it to be the previously characterized recombination signal sequence binding protein Jκ, a widely expressed and highly conserved protein that probably acts as a key adapter for transcription regulatory factors of cellular genes (Grossman *et al.*, 1994; Henkel *et al.*, 1994). Additional protein binding sites are present in the EBNA-2-responsive elements. Mutation of the PU.1 binding site has a profound effect on the EBNA-2 responsiveness of the LMP-1 promoter (Johannsen *et al.*, 1995; Sjöblom *et al.*,

1995a,b). PU.1 is also likely to be an important factor for many of the cellular genes that are activated by EBNA-2, as it is frequently involved in B lymphocyte-specific gene transcription.

(*c*) *EBNA-3A, -3B, -3C*

EBNA-3A, -3B and -3C are encoded by three genes placed tandemly in the EBV genome. Each protein is encoded by a short and a long exon 3′ to the respective mRNA (Hennessy *et al.*, 1985, 1986; Kallin *et al.*, 1986; Joab *et al.*, 1987; Petti & Kieff, 1988; Petti *et al.*, 1988; Ricksten *et al.*, 1988). The mRNAs that encode these proteins are the least abundant EBNA mRNAs, few molecules occurring in each latently infected cell. The proteins encoded by type-1 and type-2 EBV strains are only 84, 80 and 72% identical in the predicted primary amino acid sequence (Sample *et al.*, 1990). Each protein contains different repeating polypeptide domains near their carboxy termini that are responsible for size variation in different viral isolates. These EBNAs are remarkably hydrophobic and contain repeats of hydrophobic leucines, isoleucines or valines that could facilitate homo- and heterodimerization. They are located in large nuclear clumps in the nuclear matrix, chromatin and nucleoplasmic fractions but not in the nucleolus (Petti *et al.*, 1990).

Because of their structure and sequence similarity, these proteins are likely to have similar functions in latent EBV infection and transformation. EBNA-3C was shown to up-regulate the expression of CD21 mRNA in transfected Burkitt's lymphoma cells (Wang *et al.*, 1990a) and the expression of LMP-1 in Raji cells (Allday *et al.*, 1993; Allday & Farrell, 1994), while expression of EBNA-3B was shown to correlate with up-regulation of vimentin and CD40 and down-regulation of CD77 (Silins & Sculley, 1994). EBNA-3A, -3B and -3C can inhibit EBNA-2-activated transcription, probably by their capacity to bind to Jκ (Robertson *et al.*, 1995a; Krauer *et al.*, 1996; Robertson *et al.*, 1996). The complexes do not bind to DNA *in vivo*, suggesting a possible mechanism by which these proteins could modulate B-cell activation.

(*d*) *EBNA-1*

EBNA-1 is the only EBNA that is associated with chromosomes during mitosis (Reedman *et al.*, 1974; Ohno *et al.*, 1977; Grogan *et al.*, 1983). The predicted sequence of the prototype EBV type 1 strain consists of 641 amino acids that form four obvious domains: an amino terminus of 89 amino acids rich in basic residues, a 239-amino acid Gly-Ala copolymer that could form β sheets and may participate in intermolecular interactions, a short basic domain and a long hydrophobic domain from residues 459–607 which have sequence-specific DNA binding and dimerization activities (Ambinder *et al.*, 1991; Shah *et al.*, 1992; Kieff, 1996). *EBNA-1* is phosphorylated on serine residues in the carboxy terminus of the molecule (Hearing & Levine, 1985; Polvino-Bodnar *et al.*, 1988).

EBNA-1 binds to DNA by recognizing the partial palindrome TAGGATAGCATA-TGCTACCCAGATCCAG that is found at three sites in the EBV genome (Rawlins *et al.*, 1985; Ambinder *et al.*, 1990; Kieff, 1996). The site with the greatest affinity consists of 20 tandem direct repeats of the cognate sequence about 7 kb from the left end

of the genome. The second highest affinity site is 1 kb to the right and consists of two cognate sequences in dyad symmetry and two in tandem. The third site is in the *Bam*HI Q fragment about 10 kb downstream of the *EBNA-2* coding exon (Rawlins *et al.*, 1985). This site is composed of two divergent tandem repeats and appears to be important for negative regulation of the alternative promoter for latent *EBNA-1* transcription in *Bam*HI FQ (Lear *et al.*, 1992; Sample *et al.*, 1992). Binding of *EBNA-1* to the tandem repeat and dyad symmetry sites enables covalently closed, circular DNA molecules to replicate and persist as episomes (Reisman *et al.*, 1985; Yates *et al.*, 1985; Yates & Guan, 1991). The EBV DNA segment containing both sites is therefore designated *oriP*, for origin of plasmid DNA replication. The tandem repeat component acts as an *EBNA-1*-dependent enhancer on heterologous or neighbouring EBV promoters in transient transfection assays (Reisman & Sugden, 1986). The dyad symmetry component is stringently required for episome replication. The structure is partially denatured as a consequence of *EBNA-1* binding, forming bubble and cruciform structures that are characteristic of other efficient origins (Williams & Kowalski, 1993). The interaction of *EBNA-1* with the tandem repeat and dyad symmetry sites is cooperative and results in higher-order structures that lead to bending of the DNA, distortion of the duplex and looping out of the intervening sequences (Frappier & O'Donnell, 1991; Orlowski & Miller, 1991; Frappier & O'Donnell, 1992). The amino terminal of the dimerization domain is important for the formation of macromolecular complexes of *EBNA-1* homopolymers after association with the DNA templates (Goldsmith *et al.*, 1993). The carboxy terminus of *EBNA-1* determines its nuclear location by interacting with a specific protein that is homogeneously distributed on chromosomes (Ohno *et al.*, 1977; Harris *et al.*, 1985; Petti *et al.*, 1990). This property is likely to be important for segregation of episomes into progeny nuclei during mitosis. Part of *EBNA-1* is also associated with the nuclear matrix. EBNA-1 is the only EBNA that continues to be made during lytic infection. The lytic mRNA begins at a *Bam*HI F promoter near 62.2 kb (Lear *et al.*, 1992; Kieff, 1996). The two *EBNA-1* binding sites that are not part of *ori-P* are downstream of the adjacent latency I promoter in *Bam*HI Q (kb 62.3). Thus, *EBNA-1* is likely to play a role in regulation of these promoters (Sample *et al.*, 1992; Kieff, 1996).

1.1.5.3 *Latent membrane proteins*

(*a*) *LMP-1*

LMP-1 mRNA is the second most abundant viral transcript in latently infected cells (Fennewald *et al.*, 1984; Sample & Kieff, 1990). The product, encoded by three exons, is an integral membrane protein with at least three domains: a 20-amino acid hydrophilic amino terminus; six hydrophobic, 20-amino acid, alpha helical transmembrane segments separated by five reverse turns, each five to 10 amino acids in length and a 200-amino acid carboxy terminus, rich in acidic residues. The results of studies of protein cleavage in live cells are consistent with a model of plasma membrane insertion in which both the N and C termini are positioned on the cytoplasmic side and there are only three short reverse turns on the extracellular side of the membrane (Liebowitz *et al.*, 1986). At least half of LMP-1 is associated with the cytoskeleton, as defined by resistance to extraction

with nonionic detergents and co-localization with the intermediate filament protein vimentin (Mann *et al.*, 1985; Liebowitz *et al.*, 1986; Moorthy & Thorley-Lawson, 1990, 1993a). Nascent, nonionic detergent-soluble LMP-1 has a half-life of less than 2 h and is converted to an insoluble, closely cytoskeleton-associated form that is phosphorylated on serine and threonine residues in the carboxy-terminal domain (Moorthy & Thorley-Lawson, 1990, 1993a). The cytoskeletal form has a half-life in the order of 3–15 h. After phosphorylation, *LMP-1* is cleaved near the beginning of the carboxy-terminal domain, resulting in a soluble product of about 25 kDa (Moorthy & Thorley-Lawson, 1990, 1993b). LMP-1 forms patches in the cell membrane that are often organized into a single cap-like structure (Hennessy *et al.*, 1984; Liebowitz *et al.*, 1986). Although vimentin co-localizes to the patches, patch formation does not require vimentin or other EBV proteins (Wang *et al.*, 1988a; Liebowitz *et al.*, 1992) and is likely to be due to the capacity of LMP-1 to interact with itself through the transmembrane domains. Like *EBNA-1*, *LMP-1* is transcribed during lytic infection and can be induced by treatment with activators of protein kinase C (Rowe *et al.*, 1987a; Laux *et al.*, 1988a). In late lytic infection, a promoter in the third *LMP-1* exon transcribes the part of the ORF that encodes for the last two transmembrane domains and the cytoplasmic domain (Hudson *et al.*, 1985). This truncated *LMP-1* does not associate with vimentin or other cytoskeletal elements and does not have transforming or cell-activating properties (Mann *et al.*, 1985; Wang *et al.*, 1985, 1988a,b; Liebowitz *et al.*, 1992). The full-size LMP-1 is incorporated into virions (Mann *et al.*, 1985), indicating that the virion-associated products may affect the growth of newly infected cells.

Transfection of *LMP-1* with heterologous promoters has transforming effects in rodent fibroblast cell lines (Wang *et al.*, 1985; Baichwal & Sugden, 1988; Wang *et al.*, 1988a; Moorthy & Thorley-Lawson, 1993a). LMP-1 expression in Rat-1 or NIH 3T3 correlates with altered cell morphology and growth in low concentrations of serum. The cells lose anchorage dependence, acquire growth capacity in soft agar and become tumorigenic in nude mice. Expression at levels above those detected in ordinary lymphoblastoid cell lines results in toxicity (Wang *et al.*, 1988a). LMP-1 also dramatically alters the growth of EBV-negative B lymphoblasts and induces many of the changes that are usually associated with EBV infection of primary B cells, including cell clumping, increased numbers of villous projections and vimentin expression, increased surface expression of CD23, CD39, CD40, CD44, class II MHC and the cell adhesion molecules LFA-1, ICAM-1 and LFA-3 and decreased expression of CD10 (Wang *et al.*, 1988b; Birkenbach *et al.*, 1989; Wang *et al.*, 1990a; Liebowitz *et al.*, 1992; Peng & Lundgren, 1992; Zhang *et al.*, 1994a; Kieff, 1996). LMP-1 has been shown to protect B lymphocytes from apoptosis by inducing *bcl-2* (Rowe *et al.*, 1994) and probably also *A20* (Henderson *et al.*, 1991; Martin *et al.*, 1993; Fries *et al.*, 1996). In addition, LMP-1 was shown to up-regulate certain cytokines with B-cell promoting activity, such as IL-10 (Nakagomi *et al.*, 1994). *LMP-1* expression also alters the growth of multipotent haematopoietic stem cells and epithelial cells (Dawson *et al.*, 1990; Fåhraeus *et al.*, 1990a; Hu *et al.*, 1993). In monolayer cultures of immortalized human keratinocytes, LMP-1 induces morphological changes, alters cytokeratin expression and inhibits cell differen-

tiation (Dawson *et al.*, 1990; Fåhraeus *et al.*, 1990a). Similar effects were observed in the hyperplastic skin of *LMP-1* transgenic mice.

Studies with *LMP-1*-deletion mutants in rodent and human cells and the isolation of EBV recombinants specifically mutated in *LMP-1* have yielded information on the role of different protein domains in cell growth and transformation. Transfection studies with *LMP-1*-deletion mutants indicate that the cytoplasmic amino terminus is not responsible for the activating effects, the transmembrane domains are critical, probably due to their importance for LMP-1 aggregation in the plasma membrane, and the carboxy-terminal domain is essential (Wang *et al.*, 1988a,b; Baichwal & Sugden, 1989; Martin & Sugden, 1991; Liebowitz *et al.*, 1992; Moorthy & Thorley-Lawson, 1993a,b). As expected, EBV recombinants lacking *LMP*-1 were unable to induce growth transformation of primary B cells (Kaye *et al.*, 1993). Growth transformation was also abolished by deletion of progressively large parts of the amino terminal and transmembrane domains, and the same was true for deletion of all of the carboxy-terminal cytoplasmic domain. Primary B lymphocytes infected with EBV recombinants that express the entire amino terminus and transmembrane domain and the first 44 amino acids of the carboxy-terminal domain grow well on diploid fibroblast feeder layers, suggesting that at least two functional domains exist in the carboxy terminus (Kaye *et al.*, 1995). A cellular protein interaction with the first 44 amino acids of the C-terminal domain was identified in a two-hybrid screen. The LMP-1-associated protein has a ring finger and extended coiled-coil structure and is homologous to factors associated with the murine tumour necrosis factor receptor. A second LMP-1-associated protein, EBI6, is the human homologue of the murine factors. The LMP-1-associated protein binds directly not only to the LMP-1 C-terminal domain but also to the p80 tumour necrosis factor receptor, CD40 and lymphotoxin-β receptor cytoplasmic domains (Mosialos *et al.*, 1995) and to lesser extent to the cytoplasmic domains of Fas/apo-1 protein and p60 tumour necrosis factor receptor. Thus, LMP-1 seems to interact with cellular proteins that are mediators of cytoplasmic signalling from the family of tumour necrosis factor receptors and thereby induce constitutive activation of the growth, death and necrosis factor-κB signalling pathways of those receptors (Hammarskjöld & Simurda, 1992). An additional domain in the distal C-terminal end of LMP-1 seems to be involved in necrosis factor-κB activation (Huen *et al.*, 1995).

Although the standard type-1 and type-2 EBV strains are closely similar (Sample *et al.*, 1994), numerous sequence variations were identified in *LMP-1* genes from different EBV isolates. A major (*Xho*)I restriction enzyme polymorphism was detected in type-1 strains from southeast China (Hu *et al.*, 1991a,b) and in type-2 strains from Alaska. In addition, consistent nucleotide variations in the amino terminus of *LMP-1* were identified in strains marked by the *Xho*I polymorphism. Amino acids 343–352 are deleted in the Chinese strains but not in those from Alaska, and numerous other changes were detected in the amino terminus that did not co-segregate with either disease phenotype, EBV type or specific geographic region (Miller *et al.*, 1994a; see also section 1.3.2).

(*b*) *LMP-2A and -2B*

LMP-2A and -2B are encoded by spliced mRNAs transcribed from the circularized EBV genome across the terminal repeats (Laux *et al.*, 1988a). The *LMP-2A* transcription starts 3 kb downstream of the *LMP-1* polyadenylation site, whereas that of *LMP-2B* starts 0.2 kb upstream of the *LMP-1* transcription start site (Laux *et al.*, 1988a; Sample *et al.*, 1989). The *LMP-2B* and *LMP-1* promoters form a bi-directional transcription unit containing a common *EBNA-2* response element, while a separate *EBNA-2* response element regulates *LMP-2A* transcription (Zimber-Strobl *et al.*, 1993). The two proteins have unique first exons but share the remaining exons derived from the terminal repeats. These encode for 12 hydrophobic transmembrane sequences separated by short reverse turns and a 27-amino acid hydrophilic C-terminal domain. LMP-2 co-localizes with LMP-1 in the plasma membrane of latently EBV-infected B lymphocytes (Longnecker & Kieff, 1990; Longnecker *et al.*, 1991).

1.1.5.4 *Other latent viral genes*

BHRF1 (Austin *et al.*, 1988) and *Bam*HI A (Brooks *et al.*, 1993a) mRNAs have been identified in strictly latently infected cells. *BHRF1* RNA and protein are abundantly expressed early in lytic infection, but the protein is not found in latently infected cells or newly infected primary B lymphocytes (Alfieri *et al.*, 1991). An ORF, *BARF0*, is contained in a highly spliced RNA originally identified from cDNA cloning of nasopharyngeal carcinoma RNA (Sadler & Raab-Traub, 1995a). Antibodies specific for the in-vitro translated polypeptide can be detected in sera from healthy people and from nasopharyngeal carcinoma patients (Gilligan *et al.*, 1991). The antisera produced to a synthetic peptide were used to identify a doublet of 30–35 kDa in immunoblots of EBV-carrying but not EBV-negative lines and in biopsy samples of nasopharyngeal carcinoma. Cellular fractionation indicates that the protein is associated with the membrane. Although its function in EBV infection is unknown, a recombinant virus carrying a deletion encompassing the *BARF0* coding region can transform lymphocytes *in vitro* (Robertson *et al.*, 1994).

1.1.5.5 *Genes of the productive viral cycle*

Only a small fraction of latently infected B lymphocytes spontaneously enters the productive cycle; thus, lytic infection is usually induced by chemicals (Luka *et al.*, 1979; Saemundsen *et al.*, 1980; Laux *et al.*, 1988b). Of the latently infected cell lines, marmoset cells tend to be more inducible than adult human lymphoblasts, and neonatal human lymphoblasts are the least inducible (Miller & Coope, 1974). A few lymphoblastoid cell lines can be induced to permit viral replication in approximately 10% of the cells. The Akata cell line, which carries an *LMP-2A*-deleted virus, can be induced by cross-linking of surface immunoglobulin (Ig) to the extent that more than 50% of the cells enter the lytic cycle (Takada, 1984; Takada & Ono, 1989). A second approach to studying viral replication is to induce the lytic cycle by superinfection of Raji cells with defective EBV from the P3HR-1 cell line (Mueller-Lantzsch *et al.*, 1980). Raji is an EBV-positive Burkitt's lymphoma line with an unusually high EBV episome copy

number. The Raji EBV genome has at least two deletions that make it defective for DNA replication and late gene expression; the cell line is, therefore, tightly latent (Polack *et al.*, 1984a). Defective virions from P3HR-1 contain rearranged DNA molecules in which the immediate early *trans*-activators of the lytic cycle are expressed after superinfection (Cho *et al.*, 1984; Miller *et al.*, 1984).

Cells that have become permissive for viral replication undergo cytoplasmic changes that are typical of herpesviruses, including margination of nuclear chromatin, synthesis of viral DNA, assembly of nucleocapsids, envelopment of the virus by budding through the inner nuclear membrane and inhibition of host macromolecular synthesis (Gergely *et al.*, 1971). Expression of the CD21 EBV receptor is down-regulated in 12-*O*-tetradecanoyl phorbol-13-acetate (TPA)-treated EBV-positive but not EBV-negative cells, probably as a consequence of binding of viral particles (Yamamoto *et al.*, 1982).

(*a*) *Immediate early genes*

Three leftward mRNAs are transcribed after P3HR-1 superinfection of Raji or surface Ig cross-linking of Akata cells in the presence of protein synthesis inhibitors. The *BZLF1*, *BRLF1* and *BI'LF4*-encoded proteins are potent *trans*-activators of early EBV gene expression (Takada & Ono, 1989; Marschall *et al.*, 1991; Kieff, 1996). Two key early promoter regulatory elements with left and right duplications that include the origins of lytic viral DNA replication are co-ordinately up-regulated by *BZLF1* and *BRLF1* (Hudewentz *et al.*, 1982; Hummel & Kieff, 1982a; Freese *et al.*, 1983; Nuebling & Mueller-Lantzsch, 1991). The two proteins acted synergistically on the bi-directional BHRF1 and BHLF1 promoter of left duplication in assays for transient transfection (Cox *et al.*, 1990). The *BSMLF1* and *BMRF1* promoters are also coordinately regulated, probably by binding to specific response elements (Holley-Guthrie *et al.*, 1990).

The R protein is a DNA sequence-specific acidic *trans*-activator that has distant homology to *c-myb* (Gruffat & Sergeant, 1994; Kieff, 1996). The Z protein varies slightly in size among EBV strains, but the sequence differences have not been investigated (Packham *et al.*, 1993). The *BZLF1* mRNA is spliced and consists of three exons (Lieberman & Berk, 1990): the first exon encodes amino acids 1–167 which include the *trans*-activating domain (Taylor *et al.*, 1991; Chi & Carey, 1993); the second encodes amino acids 168–202 which include a strong basic domain that has homology to a conserved region of the *c-jun/c-fos* family of transcription modulators (Chang *et al.*, 1990; Lieberman & Berk, 1990) and confers the ability to interact with AP1-related sites in DNA and also targets Z to the nucleus (Mikaélian *et al.*, 1993); the third exon encodes amino acids 203–245 which include a perfect leucine or isoleucine heptad repeat capable of coiled-coil dimer formation (Chang *et al.*, 1990; Kouzarides *et al.*, 1991). This domain is required for interaction of *Z* with *p53* (Zhang *et al.*, 1994b). Over-expression of wild-type *p53* inhibits the ability of *Z* to disrupt viral latency. Thus, direct interaction between *Z* and *p53* may play a role in regulating the switch from latency to lytic infection. Dimerization may facilitate the interaction with templates that have multiple Z responsive elements. The similarity between TPA response elements, AP1 sites and *Z* recognition sites may partially explain the capacity of TPA to activate the lytic cycle (Borras *et al.*, 1996).

(*b*) *Early genes*

The early genes are expressed when the lytic cycle is induced in the presence of inhibitors of DNA synthesis. By this criterion, at least 30 EBV mRNAs are early gene products (Hummel & Kieff, 1982a,b; Baer *et al.*, 1984). Because of the difficulty in studying EBV replicative functions, proteins encoded by early genes have been identified or assigned functions by analysis of their predicted sequences or by conducting functional assays of proteins translated *in vitro* or expressed *in vivo* (Wong & Levine, 1986; Nuebling & Mueller-Lantzsch, 1991). In many cases, functions have been suggested from comparison with the primary amino-acid sequence of the herpesvirus proteins of known function. Two very abundant early proteins have been mapped to specific DNA sequences. The BALF2 protein is homologous to the HSV DNA binding protein ICP8 and is important in DNA replication (Hummel & Kieff, 1982a; Kieff, 1996). The BHRF1 protein, which is expressed in moderate abundance, has extensive collinear homology with bcl-2 (Pearson *et al.*, 1983a; Austin *et al.*, 1988). BHRF1 can protect EBV-negative Burkitt's lymphoma cells from apoptosis (McCarthy *et al.*, 1996); however, EBV recombinants lacking the *BHRF1* ORF are fully able to initiate and maintain cell growth transformation and they can also enter the lytic cycle and produce virus (Lee & Yates, 1992; Marchini *et al.*, 1991).

Several of the early genes are linked to DNA replication. These include the genes for DNA polymerase (*BALF5*), the major DNA binding protein (*BALF2*), ribonucleotide reductase (*BORF2* and *BARF1*), thymidine kinase (*BXLF1*) and alkaline exonuclease (*BGLF5*), which are distributed through the unique long domain of EBV DNA (Kieff, 1996). The ribonucleotide reductase is confined to multiple discrete regions in the cytoplasm of productively infected cells (Pearson *et al.*, 1983b, 1987). Acetone fixation destroys its immunological reactivity, suggesting that it may be a major component of the restricted early antigen complex originally identified in EBV-immune sera from African Burkitt's lymphoma patients. Transfection experiments demonstrate that some of these genes are activated in the process of cell differentiation in the absence of other viral gene products, suggesting a possible role of cellular factors in regulating the productive cycle, at least in certain cell types (Marschall *et al.*, 1991).

(*c*) *Late genes*

The late genes with partially known products or which can be identified by homology with other herpesviruses code for structural glycoproteins or proteins that modify the infected cells in order to permit viral envelopment or egress. Among the non-glycoproteins, the major nucleocapsid protein is probably encoded by *cLF1*, *NRF1* encodes the major external non-glycoprotein of the virion, and *BXRF1* is likely to encode a basic core protein (Kieff, 1996). The *BFRF3* ORF encodes a highly basic viral structural capsid protein or tegument protein, VCA p18, which is strongly immunogenic in humans (van Grunsven *et al.*, 1994). The known EBV glycoprotein genes are *BLLF1* (gp350/220), *BALF4* (gp110), *BXLF2* (gp85), *BILF2* (gp55/80), *BDLF3* (gp100–150) and *BZLF2* (gp42) (see Table 1) (Hummel *et al.*, 1984; Beisel *et al.*, 1985; Gong *et al.*, 1987; Heineman *et al.*, 1988; Oba & Hutt-Fletcher, 1988; Li *et al.*, 1995a; Nolan & Morgan, 1995; Kieff, 1996). Gp110 is one of the most abundant late EBV proteins (Emini *et al.*,

1987; Gong *et al.*, 1987; Gong & Kieff, 1990). Immune light microscopy and electron microscopy have been used to localize gp110 to the inner and outer nuclear membrane and to the cytoplasmic membranes frequently surrounding enveloped virus but not to the Golgi or plasma membrane, suggesting that the protein is not processed in the Golgi apparatus. In contrast, gp85 and gp350/220 are processed efficiently through the Golgi apparatus and are found on the virus and in the plasma membrane of lytically infected cells (Gong & Kieff, 1990; Kieff, 1996). Gp85 is a relatively minor viral component that appears to be important in fusion between the virus and cell membranes (Miller & Hutt-Fletcher, 1988). Viral penetration involves two other EBV glycoproteins, gp25 and gp42, which form a tripartite complex with gp85. Gp42 also binds to HLA DR; thus, MHC class II molecules may serve as cofactors in human B-cell infection (Li *et al.*, 1997). Gp350/220 is the dominant external viral protein involved in mediating viral binding to the B-lymphocyte receptor CD21 and is the major target of the human EBV neutralizing antibody response (Thorley-Lawson & Geilinger, 1980; Thorley-Lawson & Poodry, 1982; Tanner *et al.*, 1988; Zhang *et al.*, 1991). Injection of purified gp350/220 or infection with vaccinia recombinants that express this protein protects cotton-topped tamarins against a lethal, lymphomagenic EBV challenge (Morgan *et al.*, 1988a,b).

The late *BCRF1* gene, which is located in the middle of the EBNA regulatory domain between *ori-P* and the Cp, is a close homologue of the human *IL-10* gene, with nearly 90% collinear identity in amino-acid sequence (Moore *et al.*, 1990; Vieira *et al.*, 1991; Touitou *et al.*, 1996). *BCRF1* has most of the activities of human *IL-10*, including negative regulation of macrophage and NK cell functions and inhibition of IF γ production. Thus, virally expressed *IL-10* may have a local effect on these responses to reactivated infection. Nonsense or deletion mutations involving *BCRF1* have no effect on the ability of EBV recombinants to initiate growth transformation or to maintain latent infection or on the ability of B lymphocytes to enter the lytic cycle *in vitro* (Swaminathan *et al.*, 1993).

1.2 Methods of detection

1.2.1 *Assays to detect antibodies to EBV*

The measurement of antibodies to EBV in biological fluids remains the major means of diagnosis of EBV infection. As discussed in section 1.2.3, distinct patterns of antibody response have been identified during primary infection, latent infection of immunocompetent carriers and viral reactivation and in various EBV-associated diseases (Evans, 1972).

1.2.1.1 *Immunofluorescence*

Immunofluorescence assays are, in general, labour-intensive and time-consuming, and experience is required to interpret the resulting patterns. Preparation of the antigen slides is also subject to batch variation. Although some commercial kits are available, their use has not been accepted universally.

Antibodies to viral capsid antigen, early antigen and membrane antigen: Detection of anti-viral capsid antigen (VCA) and anti-early antigen (EA) in serum by immunofluorescence was one of the earliest tests developed and is currently the 'gold standard' with which other EBV antibody assays are compared (Henle & Henle, 1967, 1985). Burkitt's lymphoma cell lines (e.g. Raji) superinfected with P3HR1 virus or induced with TPA and/or sodium *n*-butyrate are used as the source of VCA and EA antigens. After reaction with the test serum and its dilutions, the antibody is revealed by fluorescein isothiocyanate-conjugated, class-specific anti-human Ig. Detection of specific antibodies of different isotypes is used for the diagnosis and investigation of various EBV-associated diseases such as infectious mononucleosis (IgM), nasopharyngeal carcinoma (IgA) and Burkitt's lymphoma and Hodgkin's disease (IgG). EAs can be divided into restricted (R) and diffuse (D) forms on the basis of their pattern of distribution and sensitivity to methanol (Henle *et al.*, 1971a,b). As discussed in section 1.3.3.1, antibodies against EA(D) and EA(R) may show different prevalence patterns in different EBV-associated diseases.

In order to detect anti-membrane antigen by immunofluorescence, live cells must be used.

EBNA antibodies: Anti-EBNA antibodies are detected by the anti-human complement immunofluorescence assay first developed by Reedman and Klein (1973; Reedman *et al.*, 1974). While this test is generally used for the detection of EBNA complex antibodies, more detailed analysis is now possible using cell lines transfected with individual EBNA genes.

1.2.1.2 *Enzyme-linked immunosorbent assay*

Some of the technical difficulties associated with immunofluorescence tests have been overcome by the development of specific enzyme-linked immunosorbent assays (ELISAs). The specificity of most ELISAs used in EBV serology suffers, however, in comparison with that of immunofluorescence assays. A variety of antibodies to EBV antigens can be detected in ELISA with affinity-purified native or recombinant proteins. Serological reactions to antibody-specific epitopes have been mapped with the use of synthetic peptides. ELISAs have been developed for EBNAs, EA(D), EA(R), VCA, membrane antigen and ribonucleotide reductase (Luka *et al.*, 1984; Halprin *et al.*, 1986; Uen *et al.*, 1988; Weber *et al.*, 1996). Recombinant gp350 is used for the detection of antibodies to membrane antigen in ELISAs.

1.2.1.3 *Immunoblotting*

Lysates of EBV-positive cell lines fractionated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis are used to detect anti-EBV antibodies in western blot methods. Individual antibody polypeptides can be distinguished by their characteristic size and migration patterns.

1.2.1.4 *Complement fixation*

Soluble complement-fixing antibodies were characterized when tested against soluble, non-sedimentable, nonstructural antigens extracted from non-virus-producing cells such as Raji, and were shown to appear only months after primary EBV infection. The results of this test parallel the presence of EBNA in cases of asymptomatic infectious mononucleosis, Burkitt's lymphoma and nasopharyngeal carcinoma (Gerber & Birch, 1967; Sohier & de Thé, 1972; Vonka *et al.*, 1972).

1.2.1.5 *Functional assays*

Antibodies to EBV-encoded proteins have been detected by their capacity to neutralize viral function or to mediate lysis of virus-infected target cells. For the detection of EBV DNase neutralizing antibodies, the EBV-encoded enzyme is purified from productively infected cells or expressed as a recombinant protein (Cheng *et al.*, 1980; Stolzenberg *et al.*, 1996). The last serum dilution capable of neutralizing a given amount of the enzyme (e.g. six units) gives the anti-DNase titre. In the antibody-dependent cellular cytotoxicity assay, antibodies to EBV membrane antigen are detected by their capacity to mediate killing of ^{51}Cr-labelled, productively EBV-infected cells by Fc-receptor-positive killer cells (Chan *et al.*, 1979a; Mathew *et al.*, 1981). The significance of the functional activity of these antibodies for the control of EBV infection *in vivo* is uncertain.

1.2.2 *Detection of EBV in tissues*

While serological studies can provide indirect evidence of an association of EBV with human tumours, definite identification of EBV-associated tumours rests ultimately on the detection of viral genomes and/or viral gene products in tumour tissues. Over the years, a number of methods have become available, which can be divided broadly into those based on the extraction of nucleic acids from tissues (Southern blot hybridization and the polymerase chain reaction (PCR)) and those which detect viral genomes or gene products *in situ* in single cells (in-situ hybridization and immunohistology). Some methods are used to detect and characterize viral genomes (Southern blot hybridization, PCR, DNA in-situ hybridization), and others to detect viral gene products (reverse transcriptase (RT)-PCR, RNA in-situ hybridization, immunohistology). The latter require that the viral genome be actively expressed; it is important to realize that this is also the case for EBER in-situ hybridization (see below), which has largely replaced EBV DNA in-situ hybridization for the identification of EBV. Thus, ultimate proof that the virus is absent from a given tissue can come only through the demonstration that the viral genome is absent. The suitability of these methods for the detection of EBV in tissues will be discussed using Hodgkin's disease as an example. Hodgkin's disease is particularly suited to illustrate the advantages and disadvantages of the various techniques because of its unique pathological features (see section 2.3.1), including the scarcity of neoplastic cells, the Hodgkin and Reed-Sternberg (HRS) cells, which rarely amount to more than 2% of the total cell population in an affected lymph node and which are embedded in a

background of abundant reactive cells (Herbst & Niedobitek, 1993). This scarcity made the association between EBV and Hodgkin's disease particularly difficult to establish.

1.2.2.1 *Southern blot hybridization and the polymerase chain reaction*

Prompted by seroepidemiological studies, several groups have attempted to detect viral genomes in extracts of DNA from Hodgkin's disease tissues. Studies with nucleic acid reassociation kinetics failed to do so, probably because of the limited sensitivity of this method (zur Hausen, 1976). With the development of Southern blot hybridization, a more sensitive and specific technique became available and, when probes directed, e.g. against the *Bam*HI W internal repetitive fragment of the viral genome, were used, EBV DNA was indeed detected in approximately 20% of cases of Hodgkin's disease (Weiss *et al.*, 1987; Herbst *et al.*, 1989). Southern blot hybridization involving probes for characterization of the terminal repeat region of the viral genome was used to demonstrate that in most cases the viral genomes are present as monoclonal episomes (Raab-Traub & Flynn, 1986; Weiss *et al.*, 1987; Anagnostopoulos *et al.*, 1989; Masih *et al.*, 1991). This suggested that the virus-carrying cell population is monoclonal and implicated the HRS cells as the likely cellular source. Several groups have subsequently used PCR to detect viral genomes in Hodgkin's disease tissues. These experiments were often designed to amplify sequences from the *Bam*HI W internal repetitive fragment of the viral genome, since this promised maximal sensitivity. Using this approach, viral genomes were detected in 50–80% of biopsy samples from cases of Hodgkin's disease in western countries (Herbst *et al.*, 1990; Knecht *et al.*, 1991; Shibata *et al.*, 1991a).

Interpretation of the results obtained with Southern blot hybridization and PCR has, however, been difficult. The sensitivity of Southern blot hybridization is limited, and target DNA must be present in at least 1% of the total cell population. Furthermore, because DNA extracts from heterogeneous cell populations are analysed, the cellular source of any viral DNA detected cannot be determined unequivocally. Examination of the configuration of the terminal repeat region of the EBV genome by Southern blot hybridization can provide evidence for the clonal composition of the viral genomes. Detection of monoclonal viral genomes in DNA from clonal neoplasms indicates that the tumour cells are the cellular source of the viral DNA and that EBV infection took place before clonal expansion of the neoplastic cell population. The nature and clonal origin of HRS cells are, however, uncertain; the results of a few recent studies suggest the possibility that HRS cells may be polyclonal at least in some cases (Hummel *et al.*, 1995a; Delabie *et al.*, 1996).

Analysis of DNA extracts by PCR faces the same difficulties, which are compounded by the high sensitivity of this method. It is now clear that circulating, EBV-carrying, non-neoplastic lymphocytes are found in a variety of tissues, including Hodgkin's disease lymph nodes (Herbst *et al.*, 1992; Khan *et al.*, 1992; Niedobitek *et al.*, 1992a). Thus, the demonstration of EBV DNA in any tumour by PCR cannot be taken as evidence of viral infection in the tumour-cell population. A number of RT-PCR strategies have been devised for the analysis of EBV gene expression in peripheral blood or tissues (Raab-Traub *et al*,. 1991; Brooks *et al.*, 1992; Busson *et al.*, 1992a; Qu & Rowe, 1992; Tierney

et al., 1994). With the possible exception of the demonstration of EBER expression, these methods are not suitable for diagnostic purposes but rather represent a tool for the characterization of EBV infection under conditions in which the association with EBV is known.

1.2.2.2 *In-situ hybridization and immunohistochemistry*

The study of the relationship between EBV infection and Hodgkin's disease illustrates the necessity of identifying the cellular location of the virus. The first unequivocal demonstration of EBV within the HRS cells of Hodgkin's disease came from two independent studies in which DNA in-situ hybridization was used with ^{35}S-labelled probes. Again in these studies, the *Bam*HI W internal repetitive fragment of the viral genome was used as a target, because it promises greater sensitivity than other unique genome fragments. In both studies, EBV DNA was detected exclusively in the HRS cells of a few selected cases (Anagnostopoulos *et al.*, 1989; Weiss *et al.*, 1989a). These results were subsequently confirmed, with the use of both radioactive and non-radioactive DNA probes (Herbst *et al.*, 1990; Uccini *et al.*, 1990; Coates *et al.*, 1991a,b; Delsol *et al.*, 1992). The detection rate of DNA in-situ hybridization was always lower than that of PCR, indicating comparatively low sensitivity. Moreover, when biotin-labelled probes are used, the presence of endogenous biotin may give rise to artefactual cytoplasmic labelling (Niedobitek *et al.*, 1989a; Hawkins *et al.*, 1990; Santucci *et al.*, 1993).

A major breakthrough in the analysis of EBV infection by in-situ hybridization came with the introduction of probes specific for the small nuclear EBV-encoded RNAs, *EBER-1* and *-2* (Wu *et al.*, 1990). The EBERs are abundantly expressed in all forms of EBV latency and are therefore uniquely suitable as targets for in-situ hybridization. Moreover, because of their high copy number, EBERs can be detected with non-radioactive RNA and oligonucleotide probes, making this method suitable for many laboratories (Wu *et al.*, 1991). Because of its high sensitivity, this method allows precise determination of the incidence of EBV infection in HRS cells. Thus, EBER in-situ hybridization has revealed that EBV infection in Hodgkin's disease is not restricted to HRS cells but may occur in non-neoplastic, so-called 'bystander' lymphocytes (Herbst *et al.*, 1992; Khan *et al.*, 1992; Jiwa *et al.*, 1993a). This method has been applied in many laboratories to the study of not only Hodgkin's disease but also other malignancies, leading to the identification of a variety of new EBV-associated tumours.

In parallel with these developments, several monoclonal antibodies have become available which are suitable for the detection of EBV-encoded latent and replicative proteins in sections of routinely formalin-fixed, paraffin-embedded tissues (Rowe *et al.*, 1987b; Young *et al.*, 1989b, 1991; Grässer *et al.*, 1994). This technique has been vastly improved by the development of antigen retrieval techniques with microwave irradiation or pressure cooking (Shi *et al.*, 1997), allowing the detection of epitopes in paraffin sections masked by formalin fixation and which have been undetectable hitherto. Thus, monoclonal antibodies specific for EBNA-1, EBNA-2, LMP-1 and LMP-2A can now be used for the immunohistochemical analysis of latent EBV gene expression (Grässer *et al.*, 1994; Delecluse *et al.*, 1995a; Murray *et al.*, 1996; Niedobitek *et al.*, 1997a).

Similarly, monoclonal antibodies and probes that recognize several lytic cycle gene products, e.g. BZLF1 and EA(D), are available (Young *et al.*, 1991; Brousset *et al.*, 1992, 1993; Rowlands *et al.*, 1993; Ryon *et al.*, 1993; Niedobitek *et al.*, 1997a). Unlike transcriptional studies involving PCR or western blot analysis, immunohistochemistry allows the analysis of EBV gene expression in single cells. This is particularly relevant for the study of Hodgkin's disease because of the frequent presence of EBV-carrying, non-neoplastic lymphocytes in such tissues. With the exception of EBNA-1, however, these viral proteins are not invariably expressed in virus-associated tumours. *EBNA-1* is expressed only weakly in many cases of Hodgkin's disease and is therefore often not detectable by immunohistochemistry (Grässer *et al.*, 1994). For these reasons, immunohistology cannot substitute for in-situ hybridization for the detection of latent EBV infection. Immunohistology for the detection of LMP-1 in Hodgkin's disease represents a possible exception to this rule, since this protein has been shown to be expressed in virtually all cases (Herbst *et al.*, 1991a; Pallesen *et al.*, 1991a).

1.3 Biology of EBV infection

1.3.1 *Target tissues*

1.3.1.1 *Infection* in vitro

A characteristic feature of EBV is its ability to infect human B cells *in vitro* and to convert them into permanently growing lymphoblastoid cell lines. Several studies have shown that the virus not only immortalizes mature B cells but can also infect precursor B cells, including those with immunoglobulin heavy and light chain genes in germ-line configuration (Hansson *et al.*, 1983; Ernberg *et al.*, 1987; Gregory *et al.*, 1987a). Moreover, EBV infection of immature B cells induces the expression of 'late' lymphocyte activation antigens (CD30, CD70), as also seen in Ig-expressing lymphoblastoid cell lines (Gregory *et al.*, 1987a). This finding is consistent with the detection of EBV in $CD30^+$ HRS cells with pro-B- and pre-B-cell phenotypes and genotypes (Herbst *et al.*, 1993).

The efforts to generate in-vitro models for EBV-associated non-B-cell neoplasia have been particularly successful for T lymphocytes, in which EBV infection through CD21 or a related molecule has been achieved. Several groups have recently reported the infection of immature thymocytes with EBV. Interestingly, the viral genome in these cells appears to be maintained in linear form, without episome formation. Qp-derived EBNA-1 mRNA expression has been demonstrated in such cells, and there is a high degree of lytic cycle antigen expression (Watry *et al.*, 1991; Kaufman Paterson *et al.*,. 1995; Kelleher *et al.*, 1995). This suggests that the virus–cell interaction in these cells is fundamentally different from that in B-cell lines. A more conventional result was reported by Yoshiyama *et al.* (1995), who infected a human T-cell lymphotropic virus (HTLV)-I-carrying T-cell line with EBV. This resulted in a stable latent infection with viral episomes and expression of EBNA-1 and LMP-1, reminiscent of the type-II latency seen in many EBV-associated T-cell lymphomas. In contrast to EBV-infected B cells, up-regulation of CD23, LFA-1, LFA-3 and ICAM-1 was not observed in these cells; and in

contrast to the results obtained with immature thymocytes, lytic cycle antigens were not inducible.

In view of the frequent association of EBV with carcinomas, efforts have been made to infect epithelial cells with the virus *in vitro*; however, this has proved difficult. Some reports suggest that direct infection of nasopharyngeal and cervical epithelial cells with EBV may be possible (Sixbey *et al.*, 1986; Furukawa *et al.*, 1990; Yoshizaki *et al.*, 1994); however, in most recent studies mechanisms to facilitate this process had to be devised. One such mechanism is transfection of CR2-negative keratinocytes with the gene that encodes the B-cell EBV receptor: thus, Li *et al.* (1992) achieved transient infection of CR2-transfected human keratinocytes. Subsequently, Knox *et al.* (1996) generated stable EBV-infected subclones from such cells. In these cells, EBNA-1 and low levels of LMP-1 were expressed, reminiscent of the situation in nasopharyngeal carcinomas. Moreover, the ability of these cells to undergo terminal squamous differentiation was impaired, suggesting that stable EBV infection of epithelial cells requires an undifferentiated cellular environment.

A different approach was chosen by Sixbey and co-workers, who demonstrated that coating EBV with gp350-specific polymeric IgA could facilitate binding to and infection of a human colon carcinoma cell line which was otherwise refractory to EBV infection. The EBNA complex, EBNA-2, and lytic cycle antigens were expressed in a fraction of these cells (Sixbey & Yao, 1992). The relevance of this model for EBV infection *in vivo* is as yet uncertain.

Infection of a hepatoma cell line with EBV has been reported (Lisi *et al.*, 1995), but this model has yet to be fully characterized. Of uncertain significance also is the reported generation of precursor monocyte cell lines (Revoltella *et al.*, 1989) which are EBV DNA-positive, as demonstrated by Southern blot hybridization and expressed EBNA complex.

Lindhout *et al.* (1994) achieved infection of follicular dendritic cells with EBV, presumably mediated through CR2, generating permanently growing cell lines that express LMP-1 and EBNA-2. A report of EBV infection of mouse fibroblasts after CR2 transfection demonstrates that, at least in certain cell types, the absence of CR2 is the main obstacle for EBV infection (Ahearn *et al.*, 1988).

Yet another mechanism that may be important for EBV infection of CR2-negative cells of various lineages was suggested by the results of Bayliss and Wolf (1980, 1981), who demonstrated that EBV induces cell fusion between virus-carrying B cells and other cell types, including T cells and fibroblasts. The importance of this mechanism is suggested by the reported EBV infection of endothelial cells by co-cultivation with irradiated EBV-carrying B-cell lines (Jones *et al.*, 1995). While the mechanism of entry of the virus into endothelial cells was not elucidated in this study, it suggests that it is due to cell–cell contact. This result, together with the detection of the virus in CD21-negative tumour cells, raises the possibility that cell fusion may facilitate the entry of EBV in the absence of the C3d/EBV receptor (Bayliss & Wolf, 1980; Ahearn *et al.*, 1988).

1.3.1.2 *Infection of non-neoplastic cells* in vivo

As anticipated, it has been shown in several studies that EBV-carrying B cells are readily identifiable in lymphoid tissues during infectious mononucleosis and in persistent infection. When EBER in-situ hybridization is used, numerous EBV-infected cells are found in lymphoid tissues from patients with infectious mononucleosis, mainly in the expanded paracortical areas (Niedobitek *et al.*, 1992a; Hamilton-Dutoit & Pallesen, 1994; Anagnostopoulos *et al.*, 1995; Niedobitek *et al.*, 1997b). These cells are mostly of B-cell phenotype, and a proportion has been shown to undergo plasma-cell differentiation (Robinson *et al.*, 1981; Niedobitek *et al.*, 1992a; Anagnostopoulos *et al.*, 1995; Niedobitek *et al.*, 1997b). Significantly, the expression of lytic-cycle gene products has been reported in plasma cells during infectious mononucleosis (Anagnostopoulos *et al.*, 1995; Niedobitek *et al.*, 1997b). Small numbers of EBV-positive lymphocytes are detectable in lymphoid and other tissues as well as in peripheral blood from chronic virus carriers, again, usually in the paracortex of lymphoreticular tissues; many display the phenotype of small, resting B cells (Niedobitek *et al.*, 1992a; Deamant *et al.*, 1993; Miyashita *et al.*, 1995). The number of EBV-positive cells can increase dramatically under conditions of impaired T-cell immunity, e.g. in individuals infected with the human immunodeficiency virus (HIV). On rare occasions, expansion of EBV-infected cells in germinal-centre reactions has been observed, suggesting that at least some EBV-positive circulating B cells show physiological response to antigenic stimulation (Niedobitek *et al.*, 1992a).

The possibility of infection of other cell types by EBV is more controversial. The ability of EBV to infect T cells is demonstrated by the frequent detection of the virus in T-cell non-Hodgkin lymphomas (Pallesen *et al.*, 1991b). Moreover, EBV infection has been demonstrated in certain atypical non-neoplastic T-cell proliferations, often in association with primary infection. Kikuta *et al.* (1988) demonstrated EBV infection in $CD4^+$ T cells from a boy with chronic active EBV infection and Kawasaki disease. Yoneda *et al.* (1990) reported the detection of EBV in a transient polyclonal T-cell proliferation in a young man. Moreover, several groups have demonstrated the presence of EBV in non-neoplastic T cells and natural killer (NK) cells in patients with virus-associated haemophagocytic syndrome (Kawaguchi *et al.*, 1993; Su *et al.*, 1994; Dolezal *et al.*, 1995). In agreement with these observations, EBV infection of T cells has been reported in patients with infectious mononucleosis as well as in persistent infection (Deamant *et al.*, 1993; Anagnostopoulos *et al.*, 1995, 1996); however, Niedobitek *et al.* (1992a, 1997b) could not confirm this finding, and EBV infection of T cells in healthy individuals appears to be a rare event.

Similarly, the question of the infection of normal epithelial cells with EBV is controversial. There is unequivocal evidence that in oral hairy leukoplakia, an AIDS-associated lesion of the tongue, viral replication occurs in the differentiated upper epithelial cell layers (Greenspan *et al.*, 1985; Niedobitek *et al.*, 1991a; Thomas *et al.*, 1991a; Young *et al.*, 1991; see also section 1.2.2); however, several authors have indicated that this is not accompanied by latent EBV infection in the basal epithelial cells of the lesion, suggesting that hairy leukoplakia represents a focus of isolated EBV replication without

a detectable latent phase (Niedobitek *et al.*, 1991a; Thomas *et al.*, 1991a; Sandvej *et al.*, 1992; Murray *et al.*, 1996). Lemon *et al.* (1977) and Sixbey *et al.* (1984) reported the detection by in-situ hybridization of EBV DNA in oropharyngeal epithelial cells from patients with infectious mononucleosis and in one chronic virus carrier. More recent studies of tonsils from patients with infectious mononucleosis, however, have shown no evidence of EBV infection in the tonsillar epithelium (Niedobitek *et al.*, 1989b; Weiss & Movahed, 1989; Niedobitek *et al.*, 1997b), and Karajannis *et al.* (1997) demonstrated the presence of isolated EBV-positive B cells in throat washings from infectious mononucleosis patients in the absence of detectable virus in desquamated epithelial cells. Moreover, there is no evidence of EBV infection in epithelial cells of normal nasopharyngeal mucosa, in tissues from either healthy individuals or from the vicinity of EBV-positive nasopharyngeal carcinomas (Niedobitek *et al.*, 1991b; Sam *et al.*, 1993; Tao *et al.*, 1995). The recent description of EBV infection in nasopharyngeal carcinoma *in situ* indicates that viral infection of the nasopharyngeal mucosa takes place before the infiltrative growth of the carcinoma commences (Pathmanathan *et al.*, 1995a); however, when exactly in the pathogenic process of nasopharyngeal carcinoma this occurs is as yet unclear, and present evidence would suggest that EBV infection of normal oro- and nasopharyngeal epithelial cells is at best a rare event. Similarly, EBV infection of normal gastric mucosa has not yet been demonstrated convincingly (Rowlands *et al.*, 1993; Fukayama *et al.*, 1994; Osato & Imai, 1996).

A few studies have suggested that EBV may persist in the epithelial cells of the parotid and other salivary glands, first on the basis of reassociation kinetics and DNA in-situ hybridization studies of parotid glands from healthy individuals (Wolf *et al.*, 1984). Subsequent studies using EBV DNA in-situ hybridization and immunohistochemistry showed the presence of EBV in ductal epithelial cells of salivary and lachrymal glands from patients with Sjögren's syndrome (Pflugfelder *et al.*, 1993; Wen *et al.*, 1996). Interestingly, a difference between the results of EBV DNA and EBER in-situ hybridization was noted in one of these studies. Thus, EBV DNA was localized to lymphocytes and epithelial cells, whereas latent EBV infection was detected by EBER in-situ hybridization only in lymphocytes and not in epithelial cells. This result is difficult to explain, particularly in view of the reported absence of antigens associated with lytic EBV infection (Wen *et al.*, 1996). Other studies of salivary glands from patients with Sjögren's disease and of normal tissue adjacent to salivary gland carcinomas by EBER and EBV DNA in-situ hybridization also showed no detectable EBV infection in salivary gland epithelial cells. This issue therefore remains controversial (Hamilton-Dutoit *et al.*, 1991a; DiGuiseppe *et al.*, 1994; Hamilton-Dutoit & Pallesen, 1994; Leung *et al.*, 1995a). Interestingly, EBV-infected hepatocytes and adrenocortical epithelial cells have been demonstrated occasionally in transplant recipients and in one patient with virus-associated haemophagocytic syndrome (Randhawa *et al.*, 1992; Kikuta *et al.*, 1993). These reports suggest that the cellular tropism of EBV may be broader in patients with impaired EBV-specific immunity.

Convincing evidence of infection of normal follicular dentritic cells, endothelial cells, smooth muscle cells and other cell types has not yet been reported.

1.3.1.3 *Infection in neoplasms*

The detection of EBV in certain B-cell non-Hodgkin's lymphomas is in keeping with the known ability of the virus to infect and immortalize human B cells *in vitro*. The association of EBV with endemic Burkitt's lymphoma is well documented (Epstein *et al.*, 1964; Lenoir & Bornkamm, 1987; Magrath *et al.*, 1992). Moreover, the virus is present in the majority of B-cell lymphoproliferations that develop in immunosuppressed transplant patients and in a large proportion of AIDS-related B-cell lymphomas (Hamilton-Dutoit *et al.*, 1991b; Craig *et al.*, 1993). The presence and role of EBV in tumour cells is discussed in Section 4. EBV has been detected in T-cell non-Hodgkin's lymphomas of the $CD4^+$, $CD8^+$ and NK cell phenotypes (Harabuchi *et al.*, 1990; Su *et al.*, 1990; Pallesen *et al.*, 1993). The ability of EBV to infect a broad range of target cells within the lymphoid system has also been demonstrated by the frequent detection of the virus in HRS (Herbst *et al.*, 1991a, 1993). While the precise nature and clonal origin of HRS cells are uncertain, these cells may express B- or T-cell antigens (Herbst *et al.*, 1993; Haluska *et al.*, 1994).

The ability of EBV to infect cells of non-lymphoid lineages was first illustrated by its detection in undifferentiated nasopharyngeal carcinoma (Klein, 1979). The presence of EBV in squamous-cell nasopharyngeal carcinomas and in gastric and other adenocarcinomas further demonstrates the ability of the virus to infect variously differentiated epithelial cells (Raab-Traub *et al.*, 1987; Shibata & Weiss, 1992; Pathmanathan *et al.*, 1995b; Osato & Imai, 1996).

The virus has also been detected in tumours derived from smooth-muscle cells, i.e. in leiomyomas and leiomyosarcomas, in immunocompromised patients (Prévot *et al.*, 1994; Lee *et al.*, 1995b; McClain *et al.*, 1995). Intriguingly, in some of these cases, EBNA-2 appears to be expressed in the absence of detectable LMP-1 expression (Lee *et al.*, 1995b). Moreover, the presence of clonal EBV genomes has been reported in so-called inflammatory pseudotumours. In some of these cases, the virus-carrying cells were of smooth-muscle phenotype, while in other cases the immunophenotype was consistent with derivation from follicular dendritic cells (Arber *et al.*, 1995; Selves *et al.*, 1996a). In one case with a follicular dendritic cell phenotype, LMP-1 expression was detected by immunostaining (Selves *et al.*, 1996a).

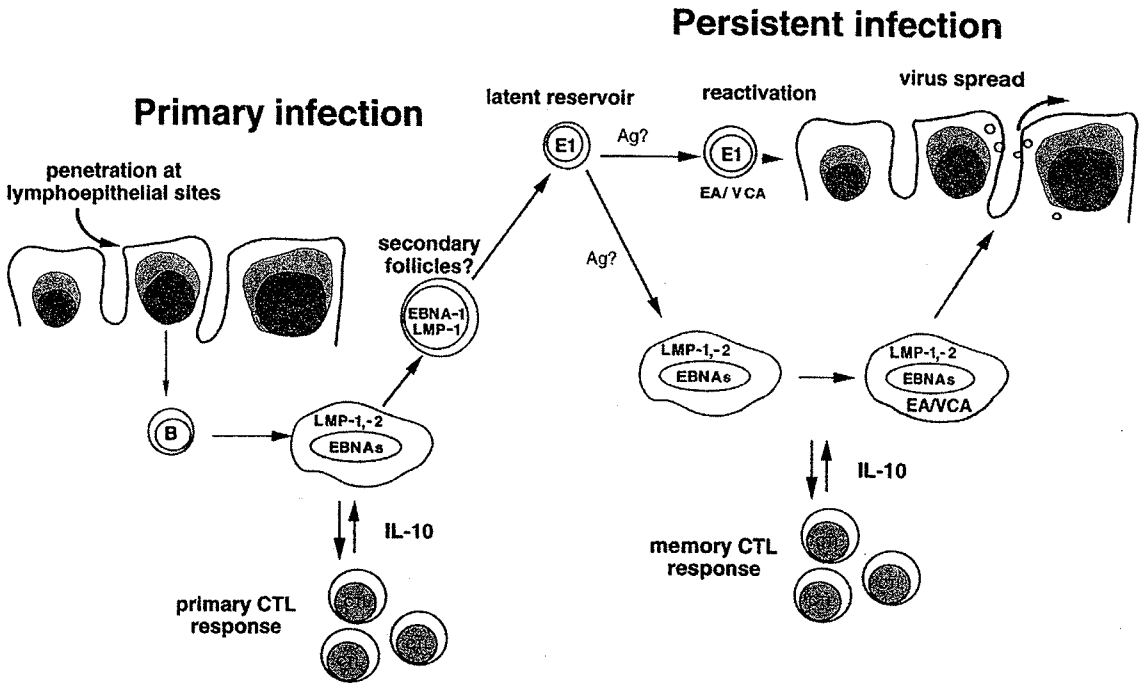
The expanding list of virus-associated tumours serves to illustrate that the target cell tropism of EBV *in vivo* is much broader than was originally anticipated. The conditions and mechanisms that allow EBV infection of these diverse cell types are, however, in most cases unknown.

1.3.2 *Persistence and latency*

Figure 2 illustrates the cellular events thought to be involved in EBV infection and persistence. Primary infection, occurring via transmission of cell-free virus and/or of productively infected cells in saliva, is associated with entry of the virus into the circulating B-cell pool. The detection of productively infected epithelial cells in the throat washings of patients in the acute phase of infectious mononucleosis has been reported (Sixbey *et al.*, 1984), and EBV can also replicate in the stratified squamous epi-

thelium lining the oral cavity, as clearly demonstrated in hairy leukoplakia lesions in immunosuppressed individuals (Greenspan *et al.*, 1985; Niedobitek *et al.*, 1991a; Lau *et al.*, 1993). It is not clear, however, whether epithelial cells are the primary targets of orally transmitted virus or whether the virus must be amplified in locally infiltrating B lymphocytes, e.g. in the tonsillary crypts. Uncertainty about the role of epithelial cells is further increased by the failure to demonstrate a putative EBV receptor on these cells. In a recent study of throat washings from infectious mononucleosis patients, EBV was not detected in desquamated epithelial cells (Karajannis *et al.*, 1997). A 200-kDa glycoprotein expressed on differentiating squamous epithelium has only some antigenic similarity to the CD21 molecule on B cells, and its presence does not correlate with EBV-binding activity (Young *et al.*, 1986; Birkenbach *et al.*, 1992). Very low levels of CD21 have been detected in some epithelial tumour lines *in vitro* (Birkenbach *et al.*, 1992) and in transplantable nasopharyngeal carcinomas, suggesting that low levels of the receptor could be expressed *in vivo*. Other routes of viral entry, such as fusion with EBV-infected, infiltrating B lymphocytes (Bayliss & Wolf, 1981) or transport as an immune complex (Sixbey & Yao, 1992), may be more relevant to epithelial-cell infection.

Figure 2. Model of EBV–host interaction during primary infection and persistence



Ag, antigen; EA, early antigen; VCA, viral capsid antigen; EBNA, EBV nuclear antigen; E1, EBV nuclear antigen 1; LMP, latent membrane protein; IL, interleukin; CTL, cytotoxic T lymphocyte

The initial events of B-cell infection *in vivo* appear to be similar to those seen on experimental infection of resting B cells *in vitro*, i.e. transcription from Wp, Cp and LMP promoters and expression of the full spectrum of latent proteins (Falk *et al.*, 1990; Tierney *et al.*, 1994). This pattern of latent viral gene expression has two important effects, both serving to increase the chances that viral persistence will be established in the lymphoid system. First, it can drive rapid polyclonal expansion of the infected B-cell

pool in the very early phases of the infection, before an effective cellular immune response has developed. Second, LMP-1 can up-regulate at least two cellular proteins, bcl-2 and A20, that are capable of extending cell survival *in vivo*. Virus-induced entry of infected cells into the long-lived B-cell pool could be important for viral persistence, since the majority of B lymphocytes are short-lived, and only a very small fraction of the newly generated cells is selected in the long-lived compartment by physiological means.

The early phase of virus-induced B-cell proliferation is curtailed by the developing T-cell response, and large numbers of infected cells appear to be eliminated by virus-specific cytotoxic T lymphocytes (CTLs) at this stage (Klein *et al.*, 1981; Svedmyr *et al.*, 1984; Callan *et al.*, 1996; Steven *et al.*, 1996), which correlates with the decrease in virus shedding in the oropharynx. Despite the strength of this primary immune response, viral latency within the B-cell pool and viral replication in the oropharynx are never completely eliminated. The B-cell compartment appears to be the true reservoir of latent virus, since pharmacological elimination of the autologous lymphoid system in patients receiving allogeneic bone-marrow transplants may result in eradication of the virus (Gratama *et al.*, 1988). Furthermore, treatment with the nucleoside analogue, acyclovir, abolished virus production in the oropharynx but had no effect on the number of latently infected cells in the blood (Yao *et al.*, 1989a). B Lymphocytes cultured from peripheral blood or lymphoid tissues show spontaneous outgrowth of lymphoblastoid cell lines, due predominantly to reactivation of viral replication and infection of co-resident B cells (Rickinson & Epstein, 1978; Rickinson *et al.*, 1978). In a very few cases, direct outgrowth of B lymphocytes latently infected *in vivo* has been suggested by the establishment of spontaneous lymphoblastoid cell lines in cultures supplemented with inhibitors of viral replication and virus-neutralizing antibodies (Lewin *et al.*, 1987, 1988). When assays for spontaneous outgrowth are performed in the presence of cyclosporin A, to prevent T-cell activation, the minimum number of cells required to be seeded for spontaneous outgrowth varies between 10^4 and 10^6 B cells per culture (von Knebel Doeberitz *et al.*, 1983; Rickinson *et al.*, 1984). Similar figures were recently obtained by semi-quantitative PCR analysis of purified B lymphocytes (Chen *et al.*, 1995a; Miyashita *et al.*, 1995). In healthy virus carriers, the same EBV strain appears to predominate in the blood and in throat washings (Gratama *et al.*, 1990, 1992). In the few cases in which detailed studies have been conducted, it was noted that individuals with large numbers of EBV-infected cells in the blood also tended to shed relatively high levels of virus in the throat (Yao *et al.*, 1985a,b). Altogether, these findings are consistent with the idea that latently infected B cells are necessary and sufficient for persistence and that epithelial infection may depend upon seeding from the lymphoid reservoir .

The persistence of EBV-infected B cells in healthy, immunocompetent virus carriers appears to involve a form of latency that is different from that seen in B cells during primary infection. PCR analysis of viral transcripts in circulating B cells from healthy donors showed expression of *EBNA-1* and *LMP-2A* mRNAs only (Qu & Rowe, 1992; Tierney *et al.*, 1994; Chen *et al.*, 1995a). Such a restricted pattern of latent viral gene expression is consistent with the conclusion that virus-carrying cells have a resting phenotype (Miyashita *et al.*, 1995) and could explain how these cells can persist in the face of efficient CTL surveillance (reviewed by Masucci & Ernberg, 1994). A switch

from the activated to the resting cell pattern of viral gene expression has never been seen to occur spontaneously in lymphoblastoid cell lines *in vitro* but can be induced experimentally by enforcing a change in cellular phenotype in cell hybrids (Contreras-Salazar *et al.*, 1989; Contreras-Brodin *et al.*, 1991; Kerr *et al.*, 1992). A similar switch might occur naturally *in vivo* if some of the infected cells could progress down a normal B-cell differentiation pathway.

Cells that express the full repertoire of growth transformation-associated antigens are likely be generated continually in asymptomatic virus carriers, since memory CTLs that are reactive against most EBNAs are maintained at high levels for life. Furthermore, some B cells must be continually triggered into the lytic cycle in order to maintain oropharyngeal shedding of infectious virus. Both events are likely to occur by reactivation from a latently infected memory B-cell pool. An interesting possibility is that both events may be governed by physiological signals that control normal B-cell behaviour. The switching of EBV-infected B cells to a blast-like phenotype and pattern of viral gene expression may therefore be triggered by conventional antigen-driven activation of the latently infected cells; similarly, physiological stimuli may be delivered to circulating B cells at mucosal surfaces which can trigger initiation of the lytic cycle.

1.3.3 *Immune responses*

1.3.3.1 *Antibody responses*

As outlined in Section 1.2, the seroepidemiology of EBV infection still relies on a set of immunofluorescence assays developed within the first decade of EBV research. These are used to measure antibody responses to the nuclear antigens (EBNA; Reedman & Klein, 1973); EA(D) and EA(R); the VCA, expressed in the cells late during the productive virus cycle (de Schryver *et al.*, 1972) and the membrane antigen, expressed on the surface of lytically infected cells (Klein *et al.*, 1969). Each of these antigens is a composite of several viral products, and detection of individual reactivity requires complex assays. Several partially successful attempts have been made to replace the fluorescence tests with ELISA-based and other assays in which recombinant proteins or synthetic peptides are used as substrates (Sternås *et al.*, 1986; Zhang *et al.*, 1991).

Studies of infectious mononucleosis patients have provided the bulk of the information available on primary antibody responses. By the time of onset of clinical symptoms, most such patients have substantial titres of IgM antibodies to VCA and rising IgG titres to both EA and VCA (Henle & Henle, 1973a; Horwitz *et al.*, 1975; Svedmyr *et al.*, 1984; Horwitz *et al.*, 1985; Marklund *et al.*, 1986; van Grunsven *et al.*, 1994). IgA antibodies to these antigens may also appear at this stage. Anti-VCA IgM disappears over the next few months, whereas anti-VCA IgG titres rise to a peak that may fall slightly and anti-EA IgG becomes either undetectable or stabilizes at a very low level. Neutralizing antibodies are detectable during the acute phase of infectious mononucleosis but only at very low titres; these increase to stable levels thereafter (Horwitz *et al.*, 1975; Lennette *et al.*, 1982). An interesting feature of the serology of infectious mononucleosis is the pattern of anti-EBNA responses. Patients in the acute phase of the disease show an IgG response to EBNA-2 (and probably also to EBNA-3A, -3B and

-3C), whereas an IgG response to EBNA-1 is not usually detectable until convalescence (Henle *et al.*, 1987). IgM antibodies to EBNA-1 are usually detected during the acute phase, but these are thought to be part of an autoantibody response with cross-reactivity to the Gly-Ala repeat domain, which EBNA-1 probably shares with several cellular proteins (Rhodes *et al.*, 1987). Anti-EBNA persists for life, whereas the persistence of other EBV antibodies, including anti-EBNA-2, varies (Henle *et al.*, 1987). The delayed antibody response to EBNA-1 is difficult to understand. The original hypothesis that it is due to delayed destruction of latently infected cells *in vivo* is difficult to reconcile with the detection of good responses to EBNA-2. One possible explanation is that EBNA-1 is somewhat protected from certain pathways of antigen processing. Alternatively, the changes in antibody reactivity between primary and persistent infection could reflect the predominance of different types of latently infected cells. In addition to EBV-specific antibodies, the early phase of infectious mononucleosis is characterized by a general increase in total IgM, IgG and IgA, which is consistent with virus-driven polyclonal activation of the B-cell system. Heterophile antibodies with the capacity to agglutinate sheep and horse erythrocytes are the basis of the Paul-Bunnel-Davidson test for the diagnosis of classic EBV-associated infectious mononucleosis (Fleisher *et al.*, 1979; Halbert *et al.*, 1982).

Healthy virus carriers consistently have antibodies to VCA, neutralizing anti-gp350 antibodies and antibodies to EBNA-1 (Henle & Henle, 1976a; Henle *et al.*, 1987). The titres can differ markedly among individuals and are usually stable over time. Only a proportion of healthy carriers have antibodies to EA or to one or more of the other EBNA proteins. Usually, there is no direct correlation between the titres of anti-VCA or anti-EA antibodies and the levels of virus replication, at least as measured by the titres of transforming virus in throat washings (Yao *et al.*, 1985a,b,c), although some individuals with extremely high titres of anti-VCA or anti-EA and low anti-EBNA-1 antibodies show signs of chronically active EBV infection (Johansson *et al.*, 1971; DuBois *et al.*, 1984; Straus *et al.*, 1985; Henle *et al.*, 1987; Miller *et al.*, 1987). The role of antibodies in maintaining persistent infection is not clear. Anti-gp350 antibodies may sensitize lytically infected cells to lysis by $CD16^+$ effectors (Pearson *et al.*, 1978a, 1979), but cannot recognize latently infected cells. Gp350 antibodies can neutralize a broad spectrum of EBV strains (Thorley-Lawson & Geilinger, 1980), however, and this may be one of the mechanisms that helps to prevent superinfection of the immune host with orally transmitted virus.

1.3.3.2 *Cell-mediated responses*

Clinically manifested primary EBV infection is associated with an unusually strong cell-mediated immune response. 'Atypical' mononuclear cells, the large numbers of which are a key feature of infectious mononucleosis, are predominantly T lymphocytes of the $CD8^+$ subset, with a small contribution of $CD4^+$ cells (Sheldon *et al.*, 1973; Svedmyr *et al.*, 1984). These activated T cells express the CD45RO marker and, when expanded *in vitro*, are programmed to die by apoptosis unless rescued by exogenously added cytokines (Moss *et al.*, 1985). The main stimulus for this response appears to be

latently infected B lymphocytes. In this context, it is possible that viral IL-10 produced at foci of lytic replication in the oropharynx serves to dampen local cellular immune responses. IL-10 also acts as a B-cell growth factor (Rousset *et al.*, 1992). Both these effects could promote spread of the virus from the initial focus of infection into the circulating B-cell pool which later elicits the generalized T-cell response.

Functional characterization of infectious mononucleosis T cells has been the subject of numerous investigations. Early observations demonstrated that freshly isolated infectious mononucleosis cells can kill a wide range of EBV-positive cells of different HLA type while not recognizing EBV-negative targets (Svedmyr & Jondal, 1975; Seeley *et al.*, 1981). This apparent lack of MHC restriction has now been resolved by the demonstration that, although $CD8^+$ CTLs with a classical pattern of MHC class I-restricted virus-specific reactivity are activated during primary infection (Strang & Rickinson, 1987a,b), these cells often exhibit cross-reactive recognition of certain allo-antigens (Schendel *et al.*, 1992; Burrows *et al.*, 1994). This appears unlikely to be a complete explanation, however, and other $CD8^+$ T-cell clones coincidentally expanded *in vivo* alongside the EBV-specific response may well contribute to the alloreactivity (Klein *et al.*, 1981). The activated $CD4^+$ population may also contain an EBV-specific component (Misko *et al.*, 1991). The magnitude of the response in acute infectious mononucleosis has led to the speculation that EBV may encode, or induce the expression of, a superantigen with the capacity to activate certain T-cell subsets in a polyclonal manner. This possibility has recently received some experimental support (Sutkowski *et al.*, 1996a,b). It has become clear that an important component of the primary EBV-specific response in infectious mononucleosis is indeed the expansion of specific CTL clones that show the same pattern of EBV antigen recognition and epitope choice as the memory response (Steven *et al.*, 1996).

A range of cell-mediated immune mechanisms is probably involved in the control of persistent EBV infection. Early work on experimentally infected cultures allowed identification of a number of activities that can delay the initial B-cell proliferation. Many of these are independent of immune status and have been ascribed to $CD16^+$ NK cells (Masucci *et al.*, 1983), to IF α release (Thorley-Lawson, 1981) or to IF γ release by $CD4^+$ T cells (Farrell *et al.*, 1997); however, these reactivities can only rarely prevent the transformation process. Complete regression of lymphoblastoid cell growth occurs specifically in cultures from virus-immune donors and is mediated *in vitro* by CTL reactivated from the pool of circulating memory T cells (Moss *et al.*, 1978, 1979; Rickinson *et al.*, 1979). The frequency of CTL precursors, estimated from regression assays, is between 1 in 10^3 and 1 in 10^4 circulating T cells (Rickinson *et al.*, 1981a). A prospective study of infectious mononucleosis patients showed that CTL memory increases during convalescence and soon reaches a stable steady state (Rickinson *et al.*, 1980a).

Techniques for expansion of reactivated CTLs, as either bulk T-cell lines or clones in IL-2-conditioned medium, have greatly facilitated analysis of their function (Wallace *et al.*, 1981, 1982a,b,c). Most of the CTL precursors are $CD8^+$ cells and recognize autologous and MHC class I-matched lymphoblastoid cells but not autologous mitogen-activated B blasts, while a much smaller fraction of the EBV-specific CTLs are $CD4^+$- and MHC class II-restricted (Rickinson *et al.*, 1980b, 1981b; Misko *et al.*, 1984). The

importance of this T-cell subpopulation in the control of latent EBV infection is supported by the finding that $CD4^+$ CTLs are associated with protective immunity in the cotton-topped tamarin model (Wilson *et al.*, 1996a). $CD4^+$ cells could also express their function by triggering Fas/apo-1 protein/Fas–ligand interaction and inducing apoptosis (Durandy *et al.*, 1997). The $CD8^+$ effectors recognize small peptide fragments of endogenously synthesized viral proteins presented on the cell surface in the peptide binding groove of HLA class I molecules. This implies that all EBV antigens constitutively expressed on lymphoblastoid cells are potential sources of immunogenic peptides. Further, since antigen presentation selects for peptides with high affinity for a particular binding groove, the identity of the dominant peptide epitopes, and hence the proteins from which they are derived, differs between individuals, depending on their HLA class I type.

Studies on a large panel of virus-immune donors, encompassing a wide range of HLA types, have shown that reactivity against epitopes from EBNA-3A, -3B and -3C forms a substantial part of the EBV-specific CTL response in a high proportion of individuals (Gavioli *et al.*, 1992; Khanna *et al.*, 1992; Murray *et al.*, 1992a; Gavioli *et al.*, 1993). To some extent, this reflects the fact that class I alleles that are common in Caucasian populations, such as A3, B7, B8 and B44, all tend to select epitopes from these proteins. In addition, certain alleles such as A11 and B44, when present, tend to be the dominant restricting determinant for EBV-specific responses and focus the reactivity to epitopes in EBNA-3B and EBNA-3C, respectively. CTL responses to EBNA-2, EBNA-LP, LMP-1 and LMP-2 have also been identified in some individuals, while class I-restricted CTLs that recognize EBNA-1-expressing cells have not been demonstrated to date. The failure to trigger EBNA-1-specific class I-restricted CTLs seems to be due to a *cis*-acting inhibitory effect of the internal Gly-Ala repeat on MHC class I-restricted presentation (Levitskaya *et al.*, 1995). While this may not necessarily prevent the triggering of CTL responses, EBNA-1-specific MHC class II-restricted CTLs were in fact demonstrated (Khanna *et al.*, 1995). The inability of the immune system to recognize EBNA-1 expressed in latently infected targets would be a major advantage to the virus in its strategy for persistence in immunocompetent hosts.

An interesting feature of the EBV system is the extent to which an individual HLA allele focuses the CTL response, not only against a single viral protein but against a single immunodominant peptide epitope within that protein. Well-documented examples of immunodominant EBV peptides and their restricting class I alleles are listed in Table 4. Rare components of the memory response can be selectively activated *in vitro* by stimulation with autologous cells that express individual EBV antigens carried by recombinant adenovirus vectors (Morgan *et al.*, 1996). The precise constraint on both peptide–HLA interaction and recognition of the complex by the T-cell receptor imply that quite small changes in peptide sequence may abrogate CTL recognition. Some CTL responses are type-specific, in that they recognize proteins encoded by type-1 but not type-2 EBV strains, while other responses recognize epitopes that are shared between EBV types (Burrows *et al.*, 1990a,b; Brooks *et al.*, 1993b; Lee *et al.*, 1993a; Hill *et al.*, 1995; Lee *et al.*, 1995a). Sequence polymorphism affecting CTL epitope regions can also occur between strains of the same type. For example, the major A11-restricted epitope in

EBNA-3B residues 416–424 is conserved in most EBV type-1 strains worldwide but is specifically mutated in all the type-1 strains from Southeast Asian populations, in whom the A11 allele itself is unusually prevalent (de Campos-Lima *et al.*, 1993a; Burrows *et al.*, 1996b). The mutated peptide sequences either do not bind to HLA A11 or, in some cases, do bind but are no longer immunogenic for T-cell responses (de Campos-Lima *et al.*, 1994). This raises the possibility that viral strains with mutations in an otherwise immunodominant epitope may have enjoyed selective advantage in a population in which the presenting allele is expressed in a high proportion of the individuals.

Table 4. Cytotoxic T lymphocyte target epitopes in latent EBV antigens

Antigen	EBV type	Amino acid residues	Cognate peptide	HLA class I restriction
EBNA-2	1	67–76	DTPLIPLTIF	B51/A2
EBNA-3A	1	158–166	QAKWRLQTL	B8
	1	325–333	FLRGRAYGL	B8
	1 and 2	596–604	SVRDRLARL	A2
	1	609–617	RLRAEAQVK	A3
	1	379–387	RPPIFIRRL	B7
	1	458–466	YPLHEQHGM	B35
EBNA-3B	1	101–115	NPTQAPVIQLVHAVY[a]	A11
	1	399–408	AVFDRKSDAK	A11
	1	416–424	IVTDFSVIK	A11
	1	481–495	LPGPQVTAVLLHEES[a]	A11
	1	551–564	DEPASTEPVHDQLL[a]	A11
	1	243–253	RRARSLSAERY	B27
EBNA-3C	1 and 2	881–889	QPRAPIRPI	B7
	1	258–266	RRIYDLIEL	B27
	1 and 2	290–299	EENLLDFVRF	B44
	1	335–343	KEHVIQNAF	B44
LMP-2A/2B	1 and 2	426–434	CLGGLLTMV	A0201
	1 and 2	329–337	LLWTLVVLL	A0201
	1 and 2	453–461	LTAGFLIFL	A0206
	1 and 2	340–349	SSCSSCPLSK	A1101
	1 and 2	419–427	TYGPVFMCL	A2404
	1 and 2	200–208	IEDPPFNSL	B40011
	1 and 2	236–244	RRRWRRLTV	B27

Adapted from de Campos-Lima *et al.* (1996)
EBNA, EBV nuclear antigen; LMP, latent membrane protein
[a]The minimal epitopes have not been identified.

Given the unique features of the in-vitro model available for the EBV system, it is not surprising that much more is known about cell-mediated immune responses to latent than to lytic infection; however, recent evidence suggests that several of the immediate and early antigens of the EBV lytic cycle can serve as targets for specific $CD8^+$ CTLs

(Bogedain *et al.*, 1995; Steven *et al.*, 1997). Cells moving through the lytic cycle may therefore be subject to a number of lytic antigen-specific responses operating either through direct cytotoxicity and/or lymphokine release. Studies with in-vitro models also show that lytically infected cells become more susceptible to lysis by NK cells (Blazar *et al.*, 1980; Patarroyo *et al.*, 1980) and, in the presence of IgG antibodies to gp350, are sensitized to antibody-dependent cellular cytotoxicity effectors (Pearson *et al.*, 1978a, 1979).

1.4 Epidemiology of EBV infection

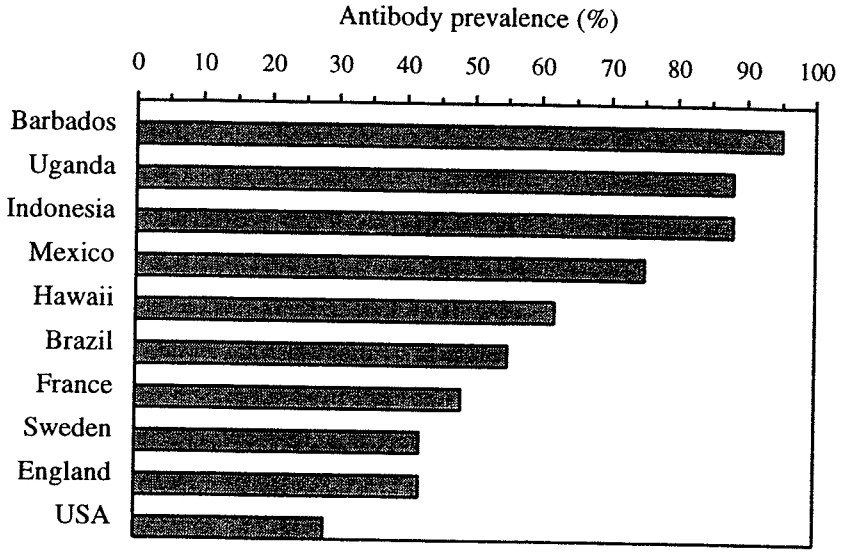
Extensive seroepidemiology studies have shown that EBV is ubiquitous throughout the world (Evans & Niederman, 1989), including the most remote populations, such as the Aleutian Islanders in Alaska (Tischendorf *et al.*, 1970) and Amazonian tribes, in whom other viral infections, such as measles, are absent (Black *et al.*, 1970).

By the third decade of life, 80–100% of individuals have become carriers of the infection. The age-specific seroprevalence of younger persons generally varies inversely with level of socioeconomic development, family size and hygienic standards. The oral route is the primary route of transmission of the virus; however, transmission by transfusion has been documented. In developing countries, infection is acquired in the first few years of life (Figure 3). Crowding and/or the practice of pre-chewing food for infants may be contributing factors. In the developed world, infection is often delayed to adolescence, when transmission is more likely because of intimate oral exposure (Evans & Niederman, 1989) (Figure 4). About 50% of primary infections during young adulthood result in clinical infectious mononucleosis (see section 1.4.1; Evans *et al.*, 1968; University Health Physicians & PHLS Laboratories, 1971; Hallee *et al.*, 1974; Gratama & Ernberg, 1995). Psychological and behavioural factors may influence the severity of clinical manifestations of EBV infections occurring after childhood (Kasl *et al.*, 1979).

The antibody response in healthy carriers is to a symptomless primary infection, as described in section 1.3.3 and reviewed recently by Evans and Kaslow (1997).

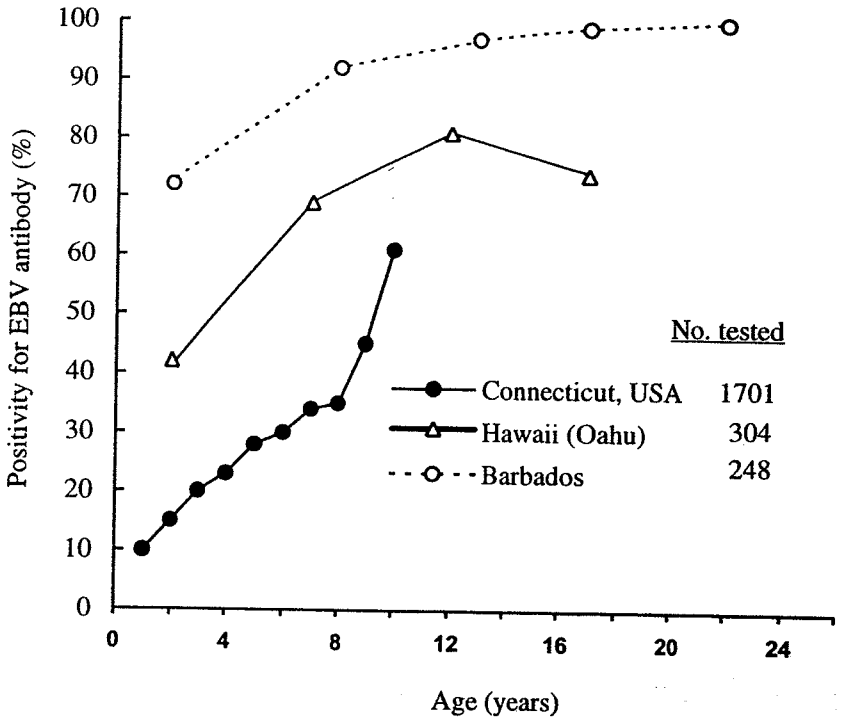
Use of molecular methods to characterize EBV genomes in human tumours led to the identification of several non-random polymorphisms, which are variably distributed between the two major EBV types and among several strains. As a result, there has been some controversy about the relationships between these polymorphisms. The major polymorphic markers identified to date and their geographic distribution are listed in Table 5. Little information is available about the incidence of these polymorphisms in the general population. On the basis of the results of a comparative study, Khanim *et al.* (1996) suggested that detection of EBV gene polymorphisms in human tumours simply reflects their overall prevalence in the respective normal population. Thus, although there is evidence from studies *in vitro* to suggest that some of these polymorphisms may be of functional relevance (see sections 4.3.1.2 and 4.5.1.6), their implication in the pathogenesis of EBV-associated tumours is not firmly established.

Figure 3. EBV antibody prevalence at age 4–6 years in various populations



From Evans & Niederman (1989)

Figure 4. Acquisition of EBV antibody by age in three areas



From Evans & Niederman (1989)

Table 5. EBV strain polymorphisms and their geographical distribution

Gene	Polymorphic marker	Goegraphical distribution	Reference
EBNA-2	Type 1 and type 2	Type 2 prevalent in central Africa and Alaska	de Campos-Lima *et al.* (1993a); Gratama & Ernberg (1995)
EBNA-3A, -3B, -3C	Type 1 and type 2	Type 2 prevalent in central Africa and Alaska	Gratama & Ernberg (1995)
EBNA-3B	Amino acid 424 K to R, T, N	South-east Asia, Papua–New Guinea	de Campos-Lima *et al.* (1993b, 1994)
EBNA-1	Amino acid 487: A, T, P, L, V	Worldwide (generated in individual virus carriers)	Bhatia *et al.* (1996); Gutiérrez *et al.* (1997)
LMP-1	C variant (loss of *Bam*HI site between W1 and I1 regions)	Southern China, Japan	Lung *et al.* (1990); Hu *et al.* (1991a); Chen *et al.* (1992a); Miller *et al.* (1994a); Gratama & Ernberg (1995)
	f variant (additional *Bam*HI site in *Bam*HI F)	Southern China	
	*Xho*I polymorphism	Southern China, Alaska	
	30-bp deletion (site loss)	China, Brazil, Africa, Europe (20–100% of the isolates)	
	33-bp repeat variation	Worldwide (generated in individual virus carriers)	
ZEBRA	Akata type (102 416 T to C mutation)	Far East	Packham *et al.* (1993)
	B95-8 type (102 743 G to A mutation)	United States, Europe, North Africa	
	Jijoye type (102 591 C to T mutation)	Central Africa	

EBNA, EBV nuclear antigen; LMP, latent membrane protein; ZEBRA, Z EBV replication activator; bp, base pair

1.5 Clinical conditions other than malignancy

1.5.1 *Infectious mononucleosis*

Infectious mononucleosis (glandular fever) is characterized clinically by fever, sore throat, tonsillitis, lymphadenopathy and splenomegaly. Tonsillitis may give rise to ulcers, leading to an erroneous diagnosis of bacterial infection. The diagnosis of infectious mononucleosis relies on the detection of atypical lymphoid cells in the peripheral blood, the occurrence of so-called heterophile antibodies and EBV seroconversion. In most cases, the disease runs its course within a few weeks, but more protracted cases are occasionally observed (Evans, 1972).

As primary EBV infection is usually asymptomatic, most of what is known about it has come from the study of patients with infectious mononucleosis and is based on the implicit assumption that asymptomatic primary EBV infection is milder than but not fundamentally different from infectious mononucleosis. This disease represents a benign lymphoproliferative disorder with prominent expansion of the paracortex of lymphoid tissues by numerous activated B blasts. Morphological, molecular and tissue culture studies have demonstrated the proliferation of EBV-infected polyclonal B blasts, accompanied by the growth of activated T cells (Svedmyr & Jondal, 1975; Tosato *et al.*, 1979; Svedmyr *et al.*, 1984; Brown *et al.*, 1986; Niedobitek *et al.*, 1992a; Anagnostopoulos *et al.*, 1995; Callan *et al.*, 1996; Niedobitek *et al.*, 1997b).

In-situ hybridization for the detection of EBERs has shown numerous latently EBV-infected cells in the paracortical areas of the tonsils of patients with infectious mononucleosis (Niedobitek *et al.*, 1992a; Anagnostopoulos *et al.*, 1995; Reynolds *et al.*, 1995; Niedobitek *et al.*, 1997b). Morphologically, these cells are a mixture, ranging from large immunoblasts, including HRS-like cells, to small lymphoid cells. Phenotypic studies in conjunction with in-situ hybridization have shown that the cells are largely B cells which often undergo plasma-cell differentiation (Anagnostopoulos *et al.*, 1995; Niedobitek *et al.*, 1997b), in keeping with the results of Robinson *et al.* (1981), who showed that during acute infectious mononucleosis up to 80% of EBNA-positive cells in peripheral blood may express cytoplasmic immunoglobulins. The detection of EBV infection in cells with evidence of plasmacytic differentiation could be due to infection of cells already committed to plasma-cell differentiation; alternatively, and more probably, this process may be secondary to EBV infection, as indicated by the demonstration that EBV infection can induce polyclonal immunoglobulin synthesis in B cells *in vitro* (Rosén *et al.*, 1977; Kirchner *et al.*, 1979). It has been suggested that plasma-cell differentiation of EBV-infected cells may in part explain the occurrence of heterophile antibodies in infectious mononucleosis patients (Robinson *et al.*, 1981).

In addition to latently EBV-infected cells, a small number of cells expressing lytic cycle antigens, such as BZLF1 and EA, are detectable in tissues from patients with infectious mononucleosis. These tend to be small lymphoid cells that are often found adjacent to crypt epithelium. Phenotypic characterization by double-labelling immunohistochemistry has shown that BZLF1-positive cells with features of plasmacellular differentiation are frequent (Niedobitek *et al.*, 1997b), confirming the results of a study showing expression of VCA in B cells with plasmacytoid differentiation *in vitro* (Crawford & Ando, 1986). Although the expression of structural viral antigens has not been analysed, it would appear that plasma cells may sustain full replication of EBV and may represent a cellular source of infectious virus in the saliva of patients with infectious mononucleosis.

Transcriptional analysis suggests that a type-III EBV latency prevails in infectious mononucleosis, with expression of the full set of EBV latent genes, including Cp/Wp-driven *EBNA-1* (Falk *et al.*, 1990; Tierney *et al.*, 1994). A more detailed analysis of EBV gene expression at the level of the single cell, reveals, however, a more heterogeneous picture. Only a subset of cells co-expresses *EBNA-2* and *LMP-1*, characteristic of type-III latency. Most cells appear to be EBER-positive but negative for EBNA-2 and LMP-1,

suggesting a type-I latency, and some large immunoblasts are seen which appear to express LMP-1 in the absence of EBNA-2 (type-II latency). Moreover, there are many small lymphocytes that express EBNA-2 but no detectable LMP-1 (Niedobitek *et al.*, 1997b). It is uncertain if this represents a new type of EBV latency or merely a transitory phenomenon. The latter possibility is supported by the observation that *EBNA-2* expression precedes that of *LMP-1* in EBV-induced B-cell immortalization *in vitro* (Alfieri *et al.*, 1991).

While infectious mononucleosis is usually a benign, self-limiting disease, complications may ensue, including rupture of the spleen when there is pronounced splenomegaly. Neurological complications, interstitial nephritis with renal failure, hepatitis, interstitial pneumonia and anaemia have also been reported (Lloyd-Still *et al.*, 1986; Imoto *et al.*, 1995; Mayer *et al.*, 1996; López-Navidad *et al.*, 1996; Morgenlander, 1996; Schuster *et al.*, 1996; Sriskandan *et al.*, 1996). The underlying mechanisms of these diseases remain uncertain. It has been suggested that such complications of acute infectious mononucleosis are due mainly to tissue infiltration by EBV-carrying B cells and reactive activated T cells or are mediated by autoantibodies (Purtilo & Sakamoto, 1981). Indeed, Sriskandan *et al.* (1996) reported recently the detection of EBV-positive lymphoid cells in the interstitium of the lungs of a patient with interstitial pneumonia after acute infectious mononucleosis. Similarly, EBV-carrying lymphocytes have been demonstrated in the portal tracts and sinusoids of the liver in patients with EBV-associated hepatitis (Markin *et al.*, 1990; Randhawa *et al.*, 1990; Hubscher *et al.*, 1994; Imoto *et al.*, 1995); however, EBV infection of occasional hepatocytes has also been reported in acute cases (Imoto *et al.*, 1995). In a renal biopsy sample from a patient with renal failure in conjunction with acute infectious mononucleosis, no EBER-positive cells were found. It has been suggested that at least some of the cases with renal impairment can be explained by a concurrent streptococcal infection causing glomerulonephritis (Mayer *et al.*, 1996).

Control of primary EBV infection and transition into a state of asymptomatic viral persistence is mediated by T-cell immunity (Rickinson & Kieff, 1996). A clonal antigen-driven expansion of $CD8^+$ T cells was recently demonstrated in acute infectious mononucleosis (Callan *et al.*, 1996). The clonal response has been shown to include virus-specific components against both latent and lytic-cycle viral antigens and to disappear during convalescence (Callan *et al.*, 1996; White *et al.*, 1996). Cases in which T-cell control of primary EBV infection fails and fatal infectious mononucleosis ensues may be due to defined genetic defects such as in X-linked lymphoproliferative syndrome. Patients with this disorder suffer from an immune defect which makes them sensitive to EBV-induced diseases; most affected men die by the age of 40 (Purtilo, 1991). Sporadic cases of fatal infectious mononucleosis without defined defects in T-cell immunity also occur. In the majority of such cases, the host appears to be overrun by excessive, uninhibited proliferation of polyclonal B cells (Falk *et al.*, 1990; Purtilo, 1991), considered to be EBV-driven on the basis of the observation of a type-III latency. In some cases, however, the proliferating B cells appear to be monoclonal, and development of morphologically malignant lymphoma has also been reported (Falk *et al.*, 1990; Purtilo, 1991; Schuster *et al.*, 1996). Thus, patients with X-linked lymphoproliferative syndrome

may develop Burkitt's lymphoma carrying *c-myc* translocations (Purtilo, 1991). Moreover, cases with transition of an infectious mononucleosis-like illness into malignant lymphoma have been reported. These include high-grade B-cell non-Hodgkin's lymphomas, acute lymphoblastic leukaemia and even cases of Hodgkin's disease (Abo *et al.*, 1982; Veltri *et al.*, 1983; Finlay *et al.*, 1986). In some of these cases, detection of EBV in the tumour cell populations was not attempted or was unsuccessful; thus, the relationship with the virus remains tenuous.

These findings suggest a scenario in which patients with impaired T-cell immunity develop massive polyclonal EBV-driven B-cell lymphoproliferation, which may show transition into classical malignant lymphoma. The observation that Burkitt's lymphomas in patients with X-linked lymphoproliferative syndrome harbour *c-myc* translocations (Purtilo, 1991) indicates that the transition from polyclonal lymphoproliferative disorder to frankly malignant lymphoma requires additional genetic changes.

1.5.2 *Oral hairy leukoplakia*

Oral hairy leukoplakia is an epithelial lesion of the tongue which was originally described in HIV-infected individuals but was subsequently also found in immunosuppressed transplant recipients. Oral hairy leukoplakia manifests itself as a raised white lesion, typically located at the lateral border of the tongue, but which may extend to other parts of the oral mucosa. Histologically, this lesion is characterized by intracellular oedema of the more superficial squamous cells, some nuclear alterations and pronounced hyperparakeratosis. While some of these changes suggested that oral hairy leukoplakia represents a human papillomavirus-induced lesion, this has not been substantiated. In 1985, EBV DNA was detected in this lesion, and the virus has been localized to the superficial epithelial cells. Linear virion DNA and the expression of viral lytic cycle antigens, e.g., BZLF1 and VCA, have been shown, indicating that epithelial cells may support EBV replication (Greenspan *et al.*, 1985; Gilligan *et al.*, 1990a; Young *et al.*, 1991). Walling *et al.* (1992) reported the presence of multiple viral strains in patients with oral hairy leukoplakia and suggested that the intense viral reproduction in this lesion contributes to the generation of EBV variants.

Morphological studies have demonstrated that the expression of lytic cycle antigens such as BZLF1 and VCA is restricted to the more differentiated upper epithelial cell layers (Greenspan *et al.*, 1985; Young *et al.*, 1991). Similarly, the expression of some latent proteins, such as EBNA-1 and LMP-1, has been reported in these cells in the context of lytic EBV infection (Thomas *et al.*, 1991a; Niedobitek *et al.*, 1991a; Sandvej *et al.*, 1992; Murray *et al.*, 1996). Current evidence suggests that EBERs are expressed at low levels, if at all (Gilligan *et al.*, 1990a; Niedobitek *et al.*, 1991a; Lau *et al.*, 1993).

In contrast to the abundance of the virus in upper epithelial cells, viral genomes and EBV gene products associated with latent infection are consistently absent from the basal or parabasal epithelial cells of oral hairy leukoplakia (Niedobitek *et al.*, 1991a; Thomas *et al.*, 1991a; Sandvej *et al.*, 1992). Together with the absence of a detectable episomal population of EBV genomes (Gilligan *et al.*, 1990a), this indicates that oral hairy leukoplakia is an isolated focus of lytic EBV infection, with no detectable latent phase.

Furthermore, oral hairy leukoplakia can be induced to regress by treatment with acyclovir, indicating that this lesion is indeed caused by EBV infection (Resnick *et al.*, 1988).

1.5.3 *The X-linked lymphoproliferative syndrome*

The X-linked lymphoproliferative syndrome is a hereditary immunodeficiency disorder characterized by a self-destructive immune response to primary EBV infection (Provisor *et al.*, 1975; Purtilo, 1976; Turner *et al.*, 1992). Patients are usually asymptomatic until they encounter EBV, but may present symptoms of immunodeficiency prior to EBV infection. After primary EBV infection, the majority of patients develop a fulminant infectious mononucleosis with a virus-associated haemophagocytic syndrome, which results in liver and bone-marrow destruction, with a fatal outcome. Patients who survive the primary infection are at high risk for developing malignant lymphoma, hypogammaglobulinaemia or aplastic anaemia. Because the immune response to EBV is ineffective, a serological response to the EBV infection may be weak or missing (Seemayer *et al.*, 1995). The *XLP* gene has been localized to Xq25 (Skare *et al.*, 1987; Sylla *et al.*, 1989), and the region spanning the smallest deletion in patients has been completely cloned (Lamartine *et al.*, 1996; Lanyi *et al.*, 1997) and sequenced; nevertheless, a good candidate gene has still to be identified. Identification of the function of the *XLP* gene is of prime importance for better understanding the complex interaction between EBV and its host.

Antiviral agents such as IF γ and α and acyclovir are ineffective, and the only curative treatment for X-linked proliferative disorder is allogeneic bone-marrow transplantation (Williams *et al.*, 1993).

1.6 Control and prevention

1.6.1 *Drugs*

Few drugs prevent replication of EBV, and even fewer are clinically useful; in fact, there are no clinically effective anti-EBV drugs with acceptable side-effects. Most of the drugs that prevent EBV replication were originally tested in studies with herpes simplex virus (HSV). The principal reagent effective against EBV is 9-(2-hydroxyethoxymethyl)-guanine, known as acyclovir when phosphorylated (Colby *et al.*, 1980; King, 1988), which is a substrate for EBV deoxynucleoside kinase and inhibits EBV DNA polymerase (King, 1988). Related compounds that are being evaluated include gancyclovir [9-(1,3-dihydroxy-2-propoxymethyl)guanine] (Cheng *et al.*, 1983), famcyclovir, phosphonoformic acid (Foscarnet) and valacyclovir (L-valyl ester of acyclovir); these are being used particularly in the management of viral infections in patients who have received bone-marrow transplants, with varying degrees of success (Dix & Wingard, 1996). Other drugs with activity against EBV include acyclic nucleoside phosphonate analogues (Lin *et al.*, 1991), a thymidine L-nucleoside (Yao *et al.*, 1996), (*E*)-5-(2-bromovinyl)- and 5-vinyl-1-β-D-arabinofuranosyluracil (Lin *et al.*, 1992), phosphorothioate oligodeoxynucleotides without sequence specification (Yao *et al.*, 1993), cucurbitane triterpenoids

(Konoshima *et al.*, 1994, 1995) and triterpenoids from ferns (Konoshima *et al.*, 1996) and quinones (Konoshima *et al.*, 1989).

A distinction should be made between those compounds that directly inhibit viral functions and those that indirectly inhibit EBV replication by interfering with interactions between host-cell components and viral components. For example, retinoic acid down-regulates *BZLF1* expression, thus indirectly reducing lytic cycle induction (Sista *et al.*, 1995), and ciclosporin A (see IARC, 1990) affects lytic cycle EBV gene expression induced by anti-immunoglobulin (Goldfeld *et al.*, 1995; Liu *et al.*, 1997). Retinoids irreversibly inhibit the growth of EBV lymphoblastoid cell lines *in vitro* by unknown mechanisms (Pomponi *et al.*, 1996). Other forms of second-messenger regulation of *BZLF1* expression undoubtedly exist (Daibata *et al.*, 1994). Induction of the EBV lytic cycle by *n*-butyrate and phorbol esters (Ito *et al.*, 1981) may also be blocked by a number of compounds; however, the effect may occur not by direct interference with EBV DNA replication but by blocking of the induction pathway activated by phorbol esters and *n*-butyrate (Daniel *et al.*, 1984; Lin & Smith, 1984). Cucurbitane and fern triterpenoids and quinones fall into this category (Konoshima *et al.*, 1989, 1994, 1995, 1996). The anti-HIV agent zidovudine (AZT) also appears to reduce EBV lytic cycle replication, but again probably by an indirect action on the intracellular environment and not directly on EBV replicative functions (Lin & Machida, 1988; Mar *et al.*, 1995).

Antiviral drugs are ineffective in the treatment of infectious mononucleosis. The clinical symptoms were not reduced by acyclovir in a number of double-blind trials. Viral excretion in throat washings was reduced in acyclovir-treated patients but resumed on cessation of treatment (Andersson & Ernberg, 1988; van der Horst *et al.*, 1991). Because EBV infection is primarily latent and only very few infected cells actually enter the lytic replicative cycle, drugs that interfere with the replication cycle are clearly of limited use in clearing EBV infection. Thus, administration of acyclovir to EBV-seropositive patients, while reducing the shedding of infectious virus in the saliva, has no effect on the level of EBV-infected circulating B lymphocytes (Yao *et al.*, 1989a,b). Treatments with acyclovir in combination with prednisolone had beneficial effects in patients with fulminant mononucleosis (Andersson & Ernberg, 1988) but had no effect on the course of less severe disease in a multicentre, placebo-controlled, double-blind trial, although oropharyngeal shedding of EBV was reduced (Tynell *et al.*, 1996). Oral hairy leukoplakia regresses on treatment with acyclovir (Resnick *et al.*, 1988), but treatment of X-linked proliferative syndrome with acyclovir has been unsuccessful (Sullivan & Woda, 1989).

The search continues for drugs that block EBV replication efficiently but have low toxicity. Their toxicity appears to be due to their incorporation into chromosomal or mitochondrial DNA. Nucleoside analogues continue to be a rich source of potentially useful compounds, the most recent being a thymidine L-nucleoside, 2′-fluoro-5-methyl-β-L-arabinofuranosyluracil, which appears to act by a different mechanism, as it is not incorporated into DNA and is of comparatively low toxicity *in vitro* (Yao *et al.*, 1996).

The EBV latency C promoter is the origin of transcripts for six EBV latent viral proteins; it is active in lymphoblastoid cell lines but silent in many EBV-associated tumours and tumour cell lines. As the C promoter is hypermethylated in these tumour lines, only *EBNA-1* is expressed. 5-Azacytidine inhibits DNA methyltransferase, resulting in demethylation of EBV genomes, activates Cp transcription and induces the expression of *EBNA-2* (Masucci *et al.*, 1989; Robertson *et al.*, 1995b). 5-Azacytidine and similar reagents may therefore render EBV tumour cells susceptible to T-cell control by inducing expression of EBV genes other than *EBNA-1* (Robertson *et al.*, 1995c). Numerous other pharmaceutical agents have been investigated that are cytotoxic to EBV tumour cells. These reagents and the effects of interferons, interleukins and RNA antisense oligomers are not considered here. Clearly, much progress will be required before drugs that are effective against EBV replication and/or transcription and are of low toxicity become available in the clinical setting.

1.6.2 *Prospects for vaccines*

The rational design of an EBV vaccine depends on an understanding of the EBV life cycle and the natural immune responses generated by the virus in humans *in vivo*. How the virus sustains a persistent latent infection in the face of a healthy immune response is not fully understood. Consequently, the development of EBV vaccines has so far been speculative. Since immunosuppressed patients are predisposed to EBV B-cell lymphomas and increased shedding of virus in the saliva (Yao *et al.*, 1985a; Thomas *et al.*, 1991b), immune control must normally exist *in vivo*. Immune responses to EBV include the generation of virus-neutralizing antibodies against envelope glycoproteins (Pearson *et al.*, 1970; de Schryver *et al.*, 1974), recognition of latent antigens by MHC class I-restricted CTLs (Rickinson *et al.*, 1980b), antibody-dependent cellular cytotoxicity against cells carrying surface gp350 (Khyatti *et al.*, 1991) and MHC class II-restricted T-cell responses (Ulaeto *et al.*, 1988; Wallace *et al.*, 1991; Lee *et al.*, 1993b; White *et al.*, 1996). Primary MHC class II-restricted CTLs have been found in seronegative people (Misko *et al.*, 1991). More recently, an EBV superantigen activity was discovered that stimulates Vβ 13 $CD4^+$ T cells from cord blood (Sutkowski *et al.*, 1996a). The various strategies adopted for EBV vaccination have changed as the understanding of the biology of the virus has evolved.

The development of a vaccine to control diseases associated with EBV must be based on the premise that most primary infections occur during the first few years of life and that sooner or later almost the entire population is exposed. The simplest view is that prevention of EBV infection, if attainable, will prevent the diseases associated with EBV, but prophylactic, post-infection and therapeutic EBV vaccination strategies should be distinguished:

- The aim of prophylactic vaccination is to prevent or modify primary infection.
- The aim of post-infection vaccination is to modify the existing immune status of an infected individual.
- Therapeutic vaccination is selectively targeted against viral antigens expressed in tumour cells.

One rationale for developing post-infection vaccines is that the rapid rise in serum IgA antibodies against lytic cycle antigens could serve as a diagnostic indicator of the onset of nasopharyngeal carcinoma and could be used to identify high-risk populations (Zeng, 1985). This marker presumably represents an increase in viral replication at some site that is linked to the emergence of the tumour. Intervention with a gp350 vaccine before this event takes place might well alter the immune balance at a crucial time and prevent development of nasopharyngeal carcinoma. Similarly, high anti-VCA titres have been used as indicators of the development of endemic Burkitt's lymphoma in Ugandan children (de Thé *et al.*, 1978a).

Therapeutic vaccination is intended to enhance the immune response against EBV-associated tumours that have already developed. In this approach, strategies involving the few EBV latent genes that are expressed in the tumours themselves, such as *EBNA-1*, *LMP-1* and *LMP-2*, would be most appropriate (Rickinson, 1995; Ambinder *et al.*, 1996; Moss *et al.*, 1996). The use of EBV latent antigen peptides representing MHC class I-restricted CTL epitopes in EBNA-3A to induce CTL memory by vaccination has recently been reviewed (Moss *et al.*, 1996). No studies on therapeutic or post-infection vaccines have yet been reported.

Current approaches to the development of prophylactic vaccines may also have applications for post-infection and therapeutic vaccines. The latter strategies have obvious target populations; the main purpose of prophylactic EBV vaccination in western countries would be to prevent infectious mononucleosis, which occurs in 50% of persons in whom EBV infection is delayed until adolescence. The incidence of infectious mononucleosis in the United States is approximately 65/100 000 (Evans, 1993), which is greater than the incidence of all other reportable diseases except gonorrhoea. Prophylactic vaccination to prevent or modify EBV infection and disease in the less developed world would require vaccination of children during the first year of life.

1.6.2.1 *Selection of an EBV vaccine antigen molecule*

Immunization can prevent tumour induction in non-human primates infected with *Herpesvirus saimiri* (Laufs & Steinke, 1975). Attenuated and killed EBV variants cannot be used as vaccines because of their oncogenic potential. The first prototype EBV vaccine was based on a purified viral envelope glycoprotein, since a correlation had been observed in serum samples between their viral neutralization activity and the reactivity of antibodies against viral membrane antigens on productively infected lymphocytes *in vitro* (Pearson *et al.*, 1970; de Schryver *et al.*, 1974). When these virus-neutralizing antibodies were used to immunoprecipitate viral glycoproteins from lysates of infected and radiolabelled cells, the viral membrane antigens were found to consist of three principal glycoprotein components (North *et al.*, 1980; Qualtière & Pearson, 1980; Thorley-Lawson & Geilinger, 1980). The larger and most abundant of the three are gp350 (gp340) and gp220, the latter being a spliced variant of the former which is encoded by the same ORF, *BLLF1* (Beisel *et al.*, 1985). Gp350 contains up to 50% carbohydrate, much of which is *O*-linked (Morgan *et al.*, 1984; Serafini-Cessi *et al.*, 1989). At least 20 ORFs in the EBV genome could potentially code for glycoproteins as

they have potential sites for *N*-linked sugars (Baer *et al.*, 1984). With the exceptions of the 110-kDa EBV counterpart of HSV gB coded for by *BALF4* (Gong & Kieff, 1990; Gong *et al.*, 1987), the product of *BILF2* (Mackett *et al.*, 1990), gp85, which is the HSV gH analogue (Heineman *et al.*, 1988; Miller & Hutt-Fletcher, 1988), and the product of *BDLF3* (Nolan & Morgan, 1995), none have been identified or characterized and certainly not evaluated for incorporation into a vaccine. Several EBV glycoprotein products will perhaps have to be incorporated into any vaccine formulation.

A number of interesting properties of gp350 have emerged since the first studies showed it to be a protective immunogen in an animal model of EBV-induced lymphoma (Epstein *et al.*, 1985). Gp350 is the virus ligand which binds to the complement receptor CD21 (Tanner *et al.*, 1987, 1988; Moore *et al.*, 1989; Birkenbach *et al.*, 1992). Cross-linking of CD21 by gp350 induces synthesis of IL-6 (Tanner *et al.*, 1996) and can modulate the synthesis of IL-1 and tumour necrosis factor (Gosselin *et al.*, 1992). CD21 is part of a membrane-signalling complex involved in the activation of B-cell immune responses, and cross-linking by gp350 may be an important early event in driving infected B cells into the cell cycle before immortalization (Sinclair & Farrell, 1995). Indeed, it might be expected that gp350 could affect other cells that express CD21, including follicular dendritic cells, T cells, monocytes and epithelial cells. These possible effects of gp350 could be both advantageous and disadvantageous in vaccination; however, the potential effects of gp350 vaccines in these respects have not been evaluated.

As serious problems have arisen in designing vaccines against HIV and influenza virus because of their capacity to generate antigenic variants, the question has arisen as to whether wild-type variation in the gp350 antigen is significant and would render a conventional vaccine based on a single laboratory strain ineffective. The sequences of *gp350* genes from several type-1 and type-2 virus isolates have been compared and no significant differences found. Furthermore, a panel of monoclonal antibodies recognizes the gp350s from all sources equally well (Lees *et al.*, 1993).

As mentioned above, the selection of a viral molecule for use as a subunit vaccine was based originally on the assumption that protective immunity would be provided by virus-neutralizing antibodies directed at glycoproteins on the surface of the virus. This is clearly not the case either in the tamarin model or the common marmoset model (see below) and is unlikely to be the only immunological criterion for protection against EBV-associated diseases in humans.

1.6.2.2 *Animal models of EBV infection, disease and vaccination*

Two primate model systems have been used in the development of a vaccine against EBV: the cotton-topped tamarin (*Saguinus oedipus oedipus*) (Miller *et al.*, 1977) and the common marmoset (*Callithrix jacchus*) (Wedderburn *et al.*, 1984; Emini *et al.*, 1986). Both have shortcomings which must be taken into account in interpreting results. Early studies showed that inoculation of EBV into the common marmoset gives rise to a poorly defined, mononucleosis-like syndrome. These observations have been extended to show that infection of the common marmoset with the M81 strain of EBV gives rise to the

long-term maintenance of antibodies to viral antigens. Furthermore, the presence of EBV DNA has now been reliably demonstrated in tissues and oral fluids of infected animals by PCR analysis (Farrell *et al.*, 1997). A crucial finding was that when infected common marmosets were paired in the same cages with uninfected animals, the uninfected animals seroconverted within four to six weeks, indicating that this animal model of EBV infection will be much more useful than previously thought (Cox *et al.*, 1996).

Injection of large doses of EBV into cotton-topped tamarins gives rise to multicentric oligoclonal B-cell lymphomas, which closely resemble those found in immunosuppressed human patients, all of which express a type-III EBV latency pattern (Cleary *et al.*, 1985; Young *et al.*, 1989b). There appears to be a threshold dose below which lymphomas do not develop. Cotton-topped tamarins have never been shown to be infected by the oral route and do not support persistent latent infection at the same level as humans, although small numbers of EBV-positive B cells have been detected in animals that have been immunized and protected from a lymphomagenic dose of EBV (Niedobitek *et al.*, 1994). This finding indicates that sterilizing immunity is not induced. The model is useful for testing the efficacy of an EBV vaccine, since protection can be achieved against a massive tumorigenic dose of virus injected intraperitoneally. This severe challenge contrasts with the normal mode of infection of humans, who receive a very small quantity of virus orally.

Other animal models have been developed for EBV-related herpesviruses. Malignant lymphomas can be induced in rabbits by an EBV-related herpesvirus from *Macaca arctoides* (Wutzler *et al.*, 1995); and a murine herpesvirus, MHV-68, although a gamma-2 herpesvirus (see Section 5 and Table 2 in the introduction) may be useful in vaccine development since it can cause lymphomas in mice and maintains a persistent infection in B cells, appearing to be a reservoir for infection *in vivo* (Nash *et al.*, 1996). Inoculation of mice with severe combined immunodeficiency with human EBV-infected lymphocytes gives rise to human B-cell lymphomas (Mosier, 1996), but this model has not yet been exploited in the development of a vaccine. [In the absence of a satisfactory animal system, it seems reasonable to progress directly to human trials, after immunogenicity and toxicity have been evaluated in animals, because of the difficulty of relating data obtained in the available animal models to human EBV infection and disease.]

1.6.2.3 *Natural gp350 subunit vaccines*

The first demonstration that gp350 is an effective subunit vaccine in the tamarin lymphoma model was obtained using material isolated from large bulk cultures of cells infected with the B95-8 laboratory isolate of EBV and induced to productive infection with sodium butyrate and phorbol esters (Morgan *et al.*, 1983). Only very small quantities of protein were isolated, which were purified by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, eluted from the gel and renatured after removal of sodium dodecyl sulfate. These small quantities of protein were incorporated into artificial liposomes made from phosphatidylcholine. Despite the small quantities of protein and the crude adjuvant system, complete protective immunity was induced in tamarins against a tumorigenic dose of EBV (Epstein *et al.*, 1985).

Gp350 prepared by mono-Q anion exchange (David & Morgan, 1988) and incorporated into immunostimulating complexes (iscoms) (Morein *et al.*, 1984; Morgan *et al.*, 1988a; Morein *et al.*, 1995) or into Syntex adjuvant formulation (Allison & Byars, 1986; Morgan *et al.*, 1989) induces protective immunity in tamarins against the standard lymphomagenic dose of virus and results in high titres of virus-neutralizing antibodies. Protective immune responses were obtained with a dose of 5 μg or less of antigen when Syntex adjuvant formulation or iscoms were used.

1.6.2.4 *Recombinant gp350 subunit vaccines*

In order to overcome the problems of yield, purity and the presence of EBV DNA, efforts have been made to express the *gp350* gene in bacteria, yeast and eukaryotic cells. Parts of the gene have been expressed in bacteria (Beisel *et al.*, 1985; Zhang *et al.*, 1991; Pither *et al.*, 1992a,b), and the complete gene has been expressed in yeast (Schultz *et al.*, 1987); however, glycosylation does not occur in bacteria, and carbohydrates different from those found on mammalian cells are added when the gene is expressed in yeast (Emini *et al.*, 1988). Emphasis was also placed on producing gp350 that could induce virus-neutralizing antibodies, since this was believed to be the key immune response required to obtain protective immunity against the virus. Although the bacterial product was recognized by antibodies from normal seropositive individuals, none of the antibodies neutralized the virus (Pither *et al.*, 1992a,b). The yeast gp350 product was not sufficiently antigenically similar, presumably because of glycosylation, and this approach was discontinued (Emini *et al.*, 1988). Cell-mediated immune responses to gp350 are known to be protective against EBV-induced lymphoma in the tamarin, however, and are likely to be important in protective immunity in humans. Since tertiary structure and glycosylation are unlikely to impede generation of the appropriate T-cell responses, both the bacterial and yeast products should be re-examined as candidate vaccines. A truncated version of *gp350* has been expressed in a baculovirus system, but the product has not been characterized in terms of its ability to induce virus-neutralizing antibodies or to induce protective immunity in the tamarin lymphoma model (Nuebling *et al.*, 1992).

The *gp350* gene has now been expressed in a number of mammalian cell systems in which glycosylation and post-translational modifications occur, which are closely similar to those found on the natural product (Motz *et al.*, 1986; Whang *et al.*, 1987; Conway *et al.*, 1989; Hessing *et al.*, 1992; Madej *et al.*, 1992). No difference has been found between these products and the natural product gp350 in terms of their ability to induce virus-neutralizing antibodies, bind a range of monoclonal antibodies and, in some studies, to stimulate the proliferation of gp350-specific T cells (Ulaeto *et al.*, 1988; Wallace *et al.*, 1991). In some studies, the membrane anchor sequence has been removed from the gene, allowing secretion of the expressed eukaryotic product into the culture medium (Whang *et al.*, 1987; Hessing *et al.*, 1992; Madej *et al.*, 1992). This approach offers major advantages in the large-scale preparation of a defined product that is relatively easy to purify. A bovine papillomavirus expression system and a Chinese hamster ovary cell system have been adapted to secrete a gp350 product in large quantities

(Hessing *et al.*, 1992; Madej *et al.*, 1992). This product is also indistinguishable from authentic gp350 in immunological terms. Another major benefit of use of this system is the guaranteed absence of potentially oncogenic EBV DNA.

1.6.2.5 *T- and B-cell epitopes on the gp350 molecule*

The generation of a panel of monoclonal antibodies (Qualtière *et al.*, 1987) allowed categorization of gp350 B-cell epitopes into at least seven groups on the basis of antibody competition. The mapping of particular immune functions to specific regions of the molecule could ultimately allow the design of a synthetic peptide or a recombinant-derived vaccine of predetermined immunological specificity. The immunological profile of gp350 is certainly complex. Most, if not all, of those epitopes associated with virus-neutralizing antibodies are discontinuous and are dependent on the conformation of the molecule, including the contribution of the large mass of carbohydrate. A possible exception is a linear epitope in the amino-terminal region between residues 317 and 327, which is recognized by antibodies from rabbits immunized either with gp350 bacterial fusion proteins containing the sequence, with intact virus or with gp350 iscoms. One approach for the identification of B- and T-cell epitopes is to express overlapping fragments of the gene in bacteria as β-galactosidase fusion proteins (Pither *et al.*, 1992a,b). The ability of antibodies made against gp350 in vaccinated animals and present in normal human sera to bind to the various fragments of gp350 expressed as bacterial fusion proteins was determined by western blotting. This procedure allows the detection only of linear epitopes, and a number of immunodominant epitopes were recognized with antisera from normal seropositive individuals. None of these epitopes was able to bind virus-neutralizing antibodies. Clear differences in the recognition patterns of sera from normal seropositive people and from immunized animals were seen, which may simply reflect species differences in immune responses but which may also reflect different immune responses after natural infection and after vaccination with a purified molecule. This difference might explain why neutralizing antibodies against linear epitopes were found in sera from vaccinated rabbits but not in sera from naturally infected humans. No method is yet available for identifying or reconstructing discontinuous B-cell epitopes, although the use of random-sequence synthetic peptides or random peptide sequences in phage libraries should be pursued (Geysen *et al.*, 1986; Cwirla *et al.*, 1990; Scott & Smith, 1990).

Gp350 bacterial fusion proteins have also been used to detect T-cell epitopes and, in some cases, to map them. It would be expected that, although the bacterial products do not retain the native conformation of gp350 and do not carry their carbohydrate complement, they should still have the capacity to be recognized by T cells after MHC class I or class II presentation. Bacterial fusion proteins were screened for their ability to induce proliferation in previously isolated gp350-specific T-cell clones from normal seropositive individuals. A large number of potential T-cell epitopes have been tentatively identified using an algorithm for this purpose, but only a few epitopes have been located in the amino-terminal region (Wallace *et al.*, 1991; Pither *et al.*, 1992a,b). Given the difficulty of identifying appropriate epitopes and the inevitable variation in

epitopes among individuals, the development of synthetic gp350 peptide vaccines seems to be remote.

1.6.2.6 *Choice of adjuvant*

Most proteins and glycoproteins are weakly immunogenic when inoculated alone into animals, and gp350 is no different in this respect. An adjuvant is invariably required to stimulate the immune response to the antigen, except when it is presented as part of a live virus or other vehicle, in which case a range of natural immune responses is triggered. A variety of effective immunological adjuvants is now available, and new ones appear from time to time (Gupta & Siber, 1995). They probably do not all work in the same way and are certainly not equally effective. Antigen presentation, targeting and delayed decomposition may all be important functions of the delivery vehicle. The mode of action of the small immuno-activator molecule may include lymphokine production, antigen processing, mitogenicity and upregulation of HLA expression. A key element in the action of adjuvants is probably their effect on the differentiation of $CD4^+$ T cells into T-helper 1 or 2 subsets after vaccination, which can influence the ratio of the two types (Dotsika *et al.*, 1997). T-helper 1 cells produce IL-2 and IF γ and enhance cell-mediated immune responses, while T-helper 2 cells produce IL-4, -5 and -10 and augment humoral immune responses (Mosmann & Coffman, 1989). It has been shown that IL-12 can substitute for certain bacterial adjuvants in enhancing cell-mediated immune responses, at least against the protozoan parasite *Leishmania major* (Afonso *et al.*, 1994). Studies of protection in the tamarin lymphoma model with natural product gp350 subunit vaccines have so far been confined to the use of artificial liposomes (North *et al.*, 1982), a threonyl derivative of muramyl dipeptide in pluronic triblock copolymers (Syntex adjuvant formulation) (Allison & Byars, 1986, 1987), iscoms (Morgan *et al.*, 1988a; Dotsika *et al.*, 1997) and aluminium salts (Finerty *et al.*, 1994).

Because some of the more powerful adjuvants have not yet been licensed for human use, studies have focused on evaluating aluminium salts, the only adjuvants licensed for use in humans, in the tamarin lymphoma model. In experiments with recombinant gp350 (Finerty *et al.*, 1994), the formulations raised protective immunity against EBV-induced lymphoma. It is worth remembering that challenge with a lymphomagenic dose of virus represents an extreme, completely unphysiological event and, in some respects, a very stringent test of the efficacy of any vaccine. Given that three out of five animals immunized with gp350 in alum were protected against lymphoma, this formulation might be sufficiently effective in humans. It also induced protective immunity in the *Callithrix jacchus* model of an EBV-induced mononucleosis-like syndrome (Emini *et al.*, 1986, 1989).

1.6.2.7 *Live virus–vector recombinants*

The disadvantage of subunit vaccines most often cited is that they generate poor and sometimes inappropriate responses, although the new generation of adjuvants mentioned above should overcome this objection. Any failure to induce a broad, powerful immune response should be set against the advantages of using biologically dead material of

absolutely defined composition (Murphy, 1989; Moss, 1996). The choice will depend on a variety of factors, including the immune responses that are required, assuming that the induction of both T- and B-cell responses is necessary together with the establishment of immunological memory. Both iscoms (Takahashi *et al.*, 1990) and Syntex adjuvant formulation induce proliferative, cytotoxic T-cell responses and memory (Byars *et al.*, 1991). Aluminium salts also allow the induction of limited cell-mediated immune responses (Dillon *et al.*, 1992).

Many recombinant viruses have been made that express one or more important vaccine molecules; so far, these have been used only rarely in humans. Gp350 has been expressed in vaccinia virus (Mackett & Arrand, 1985), adenovirus (Ragot *et el.*, 1993) and varicella virus (Lowe *et al.*, 1987). Recombinant vaccinia viruses expressing gp350 have been derived from both the relatively virulent WR laboratory strain and the attenuated Wyeth vaccine strain and both have been tested in the tamarin lymphoma model (Morgan *et al.*, 1988b). Protective immunity was induced in three of four animals by the recombinant WR strain but in none of the animals immunized with the Wyeth strain derivative. Both groups of animals responded to vaccination, with high levels of antibody against vaccinia proteins, but no antibodies against gp350 could be detected in any animal. The levels of antibodies to vaccinia proteins were substantially lower when the Wyeth derivative was used, and this is presumably a reflection of the degree to which the strain has been attenuated. The key observation in these experiments is that the WR strain derivative gives protective immunity in the absence of antibodies to gp350, so that the immunity in this case is provided by some form of cell-mediated immune response. When protective immunity was induced in the tamarin lymphoma model by injection of a replication-defective adenovirus expressing gp350, antibodies against gp350 were induced but had no capacity to neutralize EBV *in vitro* (Ragot *et al.*, 1993).

More work is needed to develop effective vaccinia recombinants which strike the correct balance between attenuation and immunogenicity. The *gp350* gene may be expressed in a canarypox vector (Taylor *et al.*, 1992) or in a vaccinia recombinant derived from the Copenhagen strain which has been specifically attenuated by the removal or inactivation of individual genes (Tartaglia *et al.*, 1992). The vaccinia gp350 recombinant previously tested in the tamarin model (Morgan *et al.*, 1988b) was also tested in the common marmoset model but with the M81 EBV strain, which resembles more closely the wild-type strains of EBV in the general population than the standard B95-8 strain. Replication of the challenge virus in the vaccinated group was reduced in comparison with control groups (Mackett *et al.*, 1996). The Oka varicella–zoster viral strain has been used to make recombinants expressing *gp350* (Lowe *et al.*, 1987), but protection was not induced in animals.

A number of recombinant adenoviruses that express foreign antigens have been constructed, and recombinant adenovirus-expressing gp350 has been tested in the tamarin model (Ragot *et al.*, 1993). Replication-defective recombinants were made in which the E1 region is deleted and the virus can be propagated only in a helper cell line which provides the deleted E1 function (Graham *et al.*, 1977). Adenoviruses have the capacity to down-regulate HLA expression in the cells they infect, but the E3 region responsible for this function has also been deleted in these replication-defective recom-

binants. Several features of adenoviruses have made them attractive for vaccine delivery. Firstly, adenoviruses type-4 and type-7 vaccines have already been used on a large scale in the United States Armed Forces to prevent respiratory disease, with a good safety record (Top *et al.*, 1971a,b). Secondly, the adenovirus can be encapsulated and given orally; although primary immune contact is in the gut lymphoid tissue, mucosal immunity is induced in the respiratory tract. Mucosal immune responses may be advantageous, but they could allow IgA-enhanced entry into epithelial cells (Sixbey & Yao, 1992).

The most significant results obtained with a recombinant virus expressing gp350 are those of a study in which the Chinese vaccinia strain (*Tien Tan*) was used to vaccinate a small group of seronegative and seropositive children in southern China (Gu *et al.*, 1995). Antibody levels to gp350 were reported to have been raised in subjects who were already seropositive and to have been induced in those who were seronegative at the beginning of the trial. Six of nine vaccinated children who were seronegative for EBV at the time of vaccination showed no evidence of natural EBV infection 16 months after vaccination.

1.6.2.8 *Cell-mediated immune responses to gp350*

Tamarins mount cell-mediated immune responses after inoculation with a lymphomagenic dose of EBV, as measured in a regression assay in which the outgrowth of autologous EBV-infected B cells was inhibited in the presence of blood lymphocytes from tamarins with spontaneously regressed EBV-induced tumours. This capacity to inhibit growth could be boosted by reinoculation of the tamarins with a sub-lymphomagenic dose of virus 18 months after the first challenge (Finerty *et al.*, 1988). More recently, EBV-specific CTLs which are $CD4^+$ MHC class II-restricted have been identified in the re-stimulated animals (Wilson *et al.*, 1996a).

Demonstration of the presence of gp350-specific T cells in vaccinated animals which can proliferate in response to gp350 *in vitro* is an immediate goal. Efforts are also being made to detect gp350-specific CTLs, but the creation of an effective target cell is a problem. Recombinant vaccinia virus expressing gp350 could be used to infect autologous fibroblasts or lymphocytes, as was done for the latent antigens (Khanna *et al.*, 1992; Murray *et al.*, 1992a). T-Cell clones specific for gp350 have been detected in normal seropositive individuals (Ulaeto *et al.*, 1988; Wallace *et al.*, 1991; Lee *et al.*, 1993b), and gp350 can stimulate the production of T cells that prevent EBV-induced transformation *in vitro* (Bejarano *et al.*, 1990). In certain circumstances, cells that express gp350 can be good targets for antibody-dependent cellular cytotoxicity (Khyatti et al., 1991). This activity is readily detected in sera from rabbits immunized with whole EBV, but its detection in sera from tamarins or rabbits vaccinated with purified gp350 has not been reported. The mechanism of cell-mediated immune protection in the tamarin lymphoma model is difficult to explain if a protective function for antibody is excluded, since protection is gp350-specific. The tumour cells themselves seem unlikely targets for gp350-specific responses as they do not express gp350 in readily detectable amounts. Cell-mediated responses induced by vaccinia–gp350 vaccination may in some way reduce the effective viral challenge dose to below the threshold required for tumour

induction. Immune responses in tamarins that had spontaneously recovered from a lymphomagenic viral challenge were possibly directed through MHC class II-restricted T cells positive for both CD4 and CD8 (Wilson *et al.*, 1996a). These cells may inhibit proliferation of lymphoblastoid cell lines by other mechanisms than perforin-mediated lysis.

1.6.2.9 *Vaccines against EBV latent antigens*

A completely different approach to EBV vaccination has emerged during the past few years, based on the knowledge that $CD8^+$ T cells are responsible, at least in part, for limiting the number of EBV-infected B lymphocytes that express most or all of the viral latency genes in the circulation of normal seropositive individuals. Could induction of these immune cells before primary infection be effective in preventing or modifying primary infection? The central problem with this approach is the same as that with gp350. How does the virus persist despite the presence of presumably effective cellular and humoral immune responses? Normal seropositive individuals have CTLs that are specific for EBNA-3A and EBNA-3C and some with activity against LMP-2 and EBNA-2. An important observation is that a large proportion of the total CTL activity in normal seropositive individuals cannot be accounted for by the EBV latent genes (Khanna *et al.*, 1992; Murray *et al.*, 1992a). The target antigens for this proportion of the CTL population have yet to be identified.

A number of epitopes that are recognized by CTLs have been located in EBNA-3A. Moss *et al.* (1996) consider that these epitopes could be used in a vaccine to elicit T-cell memory, which could then be activated to produce EBNA-3A-specific CTLs. Since the target epitope varies between HLA types, several synthetic peptides corresponding to different epitopes would have to be incorporated in such a vaccine. A relatively small number of CTL epitopes would be able to elicit an immune response in the vast majority of the population. Phase 1 trials are in progress of an EBNA-3A peptide, FLRGRAYGL, which is restricted through the HLA-B8 allele. Some circulating, latently infected B cells in bone marrow and/or the circulation, however, express only EBNA-1, which is refractory to MHC class I-restricted antigen processing (Levitskaya *et al.*, 1995; Frisan *et al.*, 1996); these B cells thus cannot be targets for MHC class I-restricted CTLs induced by either vaccination or natural infection.

1.6.2.10 *Conclusions*

The potential usefulness of gp350-based EBV vaccines depends on the roles of gp350 and lytic replication in infection and disease. Primary infection in the oropharynx, an essential stage in the infectious cycle, is mediated by gp350 in the viral envelope. Vaccine-induced mucosal IgA against gp350 could well act at this level, controlling to some extent but probably not preventing infection (Yao *et al.*, 1989a), but there is also the theoretical possibility that mucosal IgA could enhance EBV infection (Sixbey & Yao, 1992). It is impossible to know at this stage what effects gp350 vaccine-induced immune responses will have on primary EBV infection. Will it be possible to induce sterilizing immunity, or is latent infection inevitable, and, once latency has been established, will

the immune responses of the vaccinee be more effective in preventing EBV disease than those of a normal, unvaccinated, seropositive person?

Patients with infectious mononucleosis have some circulating B cells that express lytic cycle antigens (Anagnostopoulos *et al.*, 1995; Niedobitek *et al.*, 1997b), but acyclovir treatment has no effect on the course or symptoms of the disease, although virus shedding is reduced (Tynell *et al.*, 1996). It is possible that $CD4^+$ T cells primed by gp350 vaccination would be reactivated on viral challenge. Such cells could influence the course of infectious mononucleosis by inducing apoptosis of EBV-infected B cells and by down-regulating the large monoclonal or oligoclonal populations of $CD8^+$ T cells which account for much of the lymphocytosis symptomatic of infectious mononucleosis (Callan *et al.*, 1996).

The observations that the onset of nasopharyngeal carcinoma is accompanied by a rise in the titre of serum IgA antibodies against lytic cycle antigens (Zeng, 1985) and that high anti-VCA titres in Ugandan children are prognostic for the development of endemic Burkitt's lymphoma (de Thé *et al.*, 1978a) also indicate a potential role for intervention with a gp350 vaccine. Answers to the above questions will not be obtained until candidate vaccines have been evaluated in human trials. The major EBV envelope glycoprotein gp350, when expressed as a genetically engineered product in mammalian cells, could now be evaluated in trials in which the target population is first-year university students — a population at high risk of contracting infectious mononucleosis. The endpoints of a trial in this population would be EBV seroconversion, possible development of infectious mononucleosis and changes in a range of immunological parameters, such as virus-neutralizing antibodies. Any further progress in EBV vaccine development will depend heavily on the outcome of such trials.

1.6.3 *Passive immunotherapy*

As discussed in section 1.3.3, although CTLs cannot eliminate EBV from the body, they appear to be essential for maintaining control of latently infected cells. Thus, infusion of such cells should be a safe, effective form of treatment for immunodeficient patients with EBV-related lymphoproliferation. This therapeutic approach has been explored in a number of studies and clinical trials of bone-marrow transplant recipients. Papadopoulos *et al.* (1994) and Heslop *et al.* (1994) used unseparated leukocytes from EBV-seropositive donors to treat patients with EBV-associated immunoblastic lymphomas that arose after transplantation of T cell-depleted bone-marrow allografts. All of the patients responded, as judged by clinical and laboratory criteria, but some developed fatal pulmonary complications. Severe graft-versus-host disease was observed in the survivors. Thus, although adoptive transfer of unseparated leukocytes seems to be effective, this therapeutic strategy may not be generally applicable owing to the high risk of secondary complications. The problem of graft-versus-host disease has been overcome in more recent studies in which EBV-specific CTLs of donor origin reactivated *in vitro* were infused into allograft recipients (Rooney *et al.*, 1995; Heslop *et al.*, 1996). The patients did not develop complications that could be attributed to the infusion, and in three patients with signs of EBV reactivation (> 100-fold increase in EBV DNA concen-

tration measured by semiquantitative PCR), infusion of CTLs reduced the EBV load to control levels within three to four weeks. In one case, clinical resolution of the immunoblastic lymphoma was observed. Genetic marking of the infused CTLs was used to demonstrate that specific T cells that responded to challenge with virus-infected cells *in vivo* or *ex vivo* persisted for as long as 18 months. These findings support more widespread use of CTLs in the treatment of infections and cancer. Their applications may extend to other EBV-associated malignancies, such as Hodgkin's disease and nasopharyngeal carcinoma, which express a relatively restricted range of EBV-encoded antigens that could serve as target cells for CTL therapy (see Tables 3 and 6).