4. Other Data Relevant to an Evaluation of Carcinogenicity and Its Mechanisms

4.1 Growth transformation

4.1.1 *Role of EBV*

In view of the role of viral gene products in the development of non-Hodgkin's lymphoma, it is clear that an important factor is the viral gene functions that have been shown to be involved in growth transformation of normal resting human B lymphocytes (Henle *et al.*, 1967; Pope *et al.*, 1968). Growth transformation by EBV is regarded as an in-vitro equivalent of the lymphoproliferation occurring in EBV-infected individuals in the absence of a functional immune system. Growth transformation by EBV *in vitro* is defined as the induction of immortalization. EBV-infected, proliferating B lymphocytes are similar to lymphocytes activated in response to antigens, mitogens or treatment with IL-4 and anti-CD40 (Banchereau & Rousset, 1991), resulting in expression of a similar repertoire of activation markers and adhesion molecules (Åman *et al.*, 1986; Hurley & Thorley-Lawson, 1988; Alfieri *et al.*, 1991). EBV-infected tumour cells in non-Hodgkin's lymphomas *in vivo*, however, probably also have genetic and epigenetic changes, in addition to the phenotype of the immortalized B lymphocyte studied *in vitro*.

In EBV-transformed B cells, only a small number of viral genes is expressed (see section 1.1.5 for nomenclature), including those for six nuclear antigens, EBNA-1, -2, -3A, -3B, -3C and EBNA-LP, the genes for three membrane proteins, LMP-1, LMP-2A and LMP-2B (two splice variants of LMP-2), *BHRF1*, *BARF0* and two small nuclear non-polyadenylated RNAs (EBERs). *BHRF1* encodes a viral homologue of Bcl-2 (Cleary *et al.*, 1986) which is highly expressed during the lytic cycle. *BHRF1* has also been reported to be transcribed during latency in EBV-immortalized cells (Austin *et al.*, 1988; Oudejans *et al.*, 1995a), while the BHRF1 protein has not been detected in latently infected cells. *BARF0*, a reading frame contained in a set of transcripts first described in nasopharyngeal carcinomas passaged in nude mice (Hitt *et al.*, 1989; Gilligan *et al.*, 1990b, 1991), has recently been shown to encode a 30-kDa protein in lymphoblastoid cell lines and nasopharyngeal carcinoma samples (Fries *et al.*, 1997).

4.1.2 *Minimal set of transforming genes*

The transforming potential of EBV is maintained within about one-third of the viral genome (Mark & Sugden, 1982; Kempkes *et al.*, 1995a; Robertson & Kieff, 1995). Genetic analyses of recombinant viruses have also shown that not all of the genes expressed in growth-transformed cells are required for initiation and maintenance of transformation. Neither the *EBER*s (Swaminathan *et al.*, 1991) nor *EBNA-3B* (Tomkinson & Kieff, 1992a), *BARF0* (Robertson *et al.*, 1994), *EBNA-LP* (Hammerschmidt & Sugden, 1989) or *LMP-2* is required for transformation (Longnecker *et al.*, 1992; Kim & Yates, 1993). Viral *IL-10*, a gene of the lytic cycle with suspected B-cell growth factor activity, is also not required (Swaminathan *et al.*, 1993); however, *EBNA-LP* and *LMP-2* were

shown to significantly improve the outgrowth of transformed cells (Mannick *et al.*, 1991; Allan *et al.*, 1992; Brielmeier *et al.*, 1996). *EBNA-1*, *EBNA-2*, *EBNA-3A*, *-3C* and *LMP-1* are essential for initiation of transformation (Cohen *et al.*, 1989; Kaye *et al.*, 1993; Tomkinson *et al.*, 1993). *EBNA-1* (Yates *et al.*, 1984, 1985), *EBNA-2* (Kempkes et al., 1995b) and *LMP-1* (Sandberg et al., 1997) are also definitely required for maintenance of transformation, whereas *EBNA-3A* is not (Kempkes *et al.*, 1995a). Information on the role of *EBNA-3C* in the maintenance of transformation is not available. The minimal set of viral genes required for the initiation and maintenance of growth transformation is still unknown, however, and the question of whether the combination of *EBNA-1*, *EBNA-2*, *EBNA-3A*, *EBNA-3C* and *LMP-1* is also sufficient for transformation has not yet been addressed experimentally. Functional redundancy among the non-essential genes might permit the deletion of one but not several simultaneously.

4.1.3 *Growth transformation* in vitro *and induction of lymphoproliferation* in vivo

It is not known whether induction of B-cell proliferation by EBV in an immunocompromised host *in vivo* requires the same set of viral genes as EBV-induced B-cell transformation *in vitro*, or a larger or smaller set of viral genes. The genes required for growth transformation *in vitro* are obviously most likely to be involved in the induction of lymphoproliferation *in vivo* and therefore represent the main focus here. It should be kept in mind, however, that the genes required *in vitro* are not necessarily those required *in vivo*. Proliferating cells may receive signals from their surroundings that may bypass the requirement for an active viral oncogene. Alternatively, genes that are unnecessary for growth transformation i*n vitro* may be absolutely essential for the induction of lymphoproliferation *in vivo*. Given the complexity of the virus–host interaction, which has evolved over millions of years, this is the more likely possibility. The complete life cycle of the virus is influenced by its interaction with the immune system of the host *in vivo* and therefore plays an important role in the development of EBV-associated malignancies. Control of latency *in vivo*, reactivation, re-entry into the lytic cycle, reinfection of new target cells and interference by the immune system at any of these levels are therefore of key importance for EBV-induced lymphoproliferation *in vivo*. These events not only implicate the viral genes involved in growth transformation and their cellular targets but also reflect the complexity of the virus–host interaction *in vivo*, which can be studied only in a model system of a related primate virus and its natural host.

4.1.4 *Viral transcription pattern after infection of human primary B lymphocytes by EBV*

Infection of B cells with EBV is mediated through CD21, the receptor for complement component C3d (Fingeroth *et al.*, 1984). Binding of the virus triggers a signalling cascade initiated from the CD21 molecule, which activates the cell and gives it the competence to respond to the growth-promoting action of *EBNA-2* and *EBNA-LP* (Hutt-Fletcher, 1987; Sinclair *et al.*, 1994). The viral genome is circularized within about 16–20 h after infection (Hurley & Thorley-Lawson, 1988) and gives rise to large transcripts initiated in the W promoter of the large internal repeats (Woisetschlaeger *et al.*, 1990).

After expression of *EBNA-1* and *EBNA-2*, transcription switches to the C promoter and gives rise to multiply spliced transcripts that code for EBNA-LP, EBNA-2, EBNA-3A, -3B, -3C and EBNA-1 (Schlager *et al.*, 1996; Woisetschlaeger *et al.*, 1990). *OriP*, to which EBNA-1 binds, acts not only as an origin of replication (Yates *et al.*, 1984) but also as an EBNA-1-dependent enhancer of the C promoter (Sugden & Warren, 1989) and the *LMP-1* promoter (Gahn & Sugden, 1995). C promoter transcription occludes the W promoter and brings the viral genome under its own transcriptional control (Puglielli *et al.*, 1996). Expression of the *LMPs* is under transcriptional control of *EBNA-2*. *LMP-1* and *LMP-2B* are transcribed from a bi-directional promoter that responds to *EBNA-2* (Laux *et al.*, 1994a; Johannsen *et al.*, 1995), whereas *LMP-2A* is transcribed from a separate promoter controlled by *EBNA-2* (Zimber-Strobl *et al.* , 1993).

4.1.5 *Viral proteins involved in growth transformation*

4.1.5.1 *EBNA-1*

EBNA-1 is the only viral protein expressed in growth-transformed cells that binds directly to DNA in a sequence-specific manner through its DNA binding and dimerization domain in the C terminus (Ambinder *et al.*, 1991; Bochkarev *et al.*, 1995). EBNA-1 binds to the plasmid origin of replication (*oriP*) which consists of multiple tandem repeats of EBNA-1 binding sites followed by two symmetrical binding sites (Rawlins *et al.*, 1985; Reisman *et al.*, 1985). Cooperative binding of EBNA-1 to the multiple binding sites on *oriP* is required, and sufficient, for episomal replication of the viral genome. Furthermore, *oriP* acts as an EBNA-1-dependent enhancer and plays a crucial role in the regulation of viral transcription from both the C and the *LMP-1* promoter in growth-transformed cells (Sugden & Warren, 1989; Gahn & Sugden, 1995). Because of their role in episomal replication and transcription regulation, EBNA-1 and *oriP* are of prime importance for EBV-induced proliferation of lymphocytes *in vitro* and *in vivo*.

Apart from its role in regulation of viral replication and transcription, EBNA-1 might also affect cellular replication and transcription if binding sites for this protein exist in the cellular genome. Comparison of EBV-negative Burkitt's lymphoma cell lines with EBV-positive group I Burkitt's lymphoma cell lines expressing EBNA-1, however, indicates that the growth pattern and cellular phenotypes are identical (Rowe *et al.*, 1987a; Falk *et al.*, 1993).

EBNA-1 can bind RNA *in vitro* through arginine/glycine (RGG) motifs (Snudden *et al.*, 1994). EBNA-1 also activates expression of the lymphoid recombinase genes (RAGs) through an as yet unidentified mechanism (Srinivas & Sixbey, 1995). Activation of the RAGs could promote chromosomal rearrangement and translocations and possibly also facilitate viral integration. This may indicate that EBNA-1 can activate expression of critical cellular genes and affect cellular growth control. EBNA-1 is also expressed in the permissive EBV infection, hairy leukoplakia, indicating that it contributes to replicative EBV infection (Murray *et al.*, 1996).

Evidence that EBNA-1 may itself have oncogenic potential is provided by the finding that lymphomas developed in two transgenic mouse lines expressing EBNA-1 under the

control of the immunoglobulin heavy-chain intron enhancer (Wilson *et al.*, 1996a). Moreover, EBV-positive EBNA-1-expressing Akata cell clones were reported to be able to clone in soft agar and to induce tumours in nude mice (Shimizu *et al.*, 1994).

Another hallmark of EBNA-1 is an array of glycine–alanine repeats located in the N-terminal part of the protein. These repeats were shown to prevent presentation of MHC class I-restricted CTL epitopes (Levitskaya *et al.*, 1995), which represents a novel, unique mechanism for bypassing recognition by cytotoxic T cells and maintaining viral latency.

4.1.5.2 *EBNA-LP*

EBNA-2 and *EBNA-LP* are the first viral genes expressed after infection of primary human B cells with EBV (Rooney *et al.*, 1989; Allday *et al.*, 1989). *EBNA-LP* appears to be important at least for initiation of B-cell transformation, since mutant virus lacking the C-terminal exons has a severely reduced transformation potential, and cells infected with mutant virus require a feeder layer for outgrowth (Mannick *et al.*, 1991). Ectopic expression of *EBNA-LP* in these cells restored the growth behaviour (Allan *et al.*, 1992).

EBNA-LP was reported to bind to *p53* and *Rb* (Szekely *et al.*, 1993), but there is no evidence that the function of *p53* and *Rb* is modified by such binding (Allday *et al.*, 1995; Inman & Farrell, 1995). *EBNA-LP* was recently described as localizing to the nuclear compartment defined by accumulation of the promyelocytic leukaemia gene product in normal cells (Dyck *et al.*, 1994; Weis *et al.*, 1994; Szekely *et al.*, 1996), the main function of which is again unknown. A role for *EBNA-LP* in cell cycle regulation is suggested by the finding that, with *EBNA-2*, it can induce cyclin D2 and cell cycle activation in primary human B cells pretreated with gp340 (Sinclair *et al.*, 1994) and that *EBNA-LP* phosphorylation is dependent on the cell-cycle stage (Kitay & Rowe, 1996a). Recent studies suggest that *EBNA-LP* greatly enhances *EBNA-2*-induced *trans*-activation of *LMP-1* expression through interaction with the *EBNA-2* acidic *trans*-activation domain (Harada & Kieff, 1997). Expression of *EBNA-LP* in transgenic mice has no effect on development or tumour incidence; the animals die of heart failure, probably due to a toxic effect of EBNA-LP itself (Huen *et al.*, 1993).

4.1.5.3 *EBNA-2*

EBNA-2 has long been suspected to play a crucial role in growth transformation, because a 6.6-kb deletion in the P3HR1 viral strain, encompassing the boundary of the large internal repeats to the long unique region (Ragona *et al.*, 1980; Bornkamm *et al.*, 1980, 1982; Jeang & Hayward, 1983), is associated with loss of transforming capacity, while infectivity is maintained (Miller *et al.*, 1974). A recombinant transforming virus lacking the deletion was observed after either superinfection of Raji cells with P3HR1 virus (Fresen *et al.*, 1980; Skare *et al.*, 1985) or by reconstituting the deletion in the P3HR1 virus with cloned fragments spanning the deletion and harbouring the *EBNA-2* gene (Cohen *et al.*, 1989; Hammerschmidt & Sugden, 1989). Analysis of the virus harboured by the parental Jijoye line from which P3HR1 had been obtained by single cell cloning (Hinuma *et al.*, 1967) revealed the surprising finding that the *EBNA-2* genes of

Jijoye and B95-8 share only about 50% sequence homology (Dambaugh *et al.*, 1984; Adldinger *et al.*, 1985). Although strain differences between EBV types 1 (B95-8 prototype) and 2 (Jijoye and AG876 prototypes; Dambaugh *et al.*, 1984) were later also found in other genes (Sample *et al.*, 1990), the difference in the transforming ability of the two strains (Rickinson *et al.*, 1987) could be pinpointed to the differences in the *EBNA-2* genes (Cohen *et al.*, 1992).

EBNA-2 expressed from the SV40 promoter in transgenic mice induced tubular kidney hyperplasia which advanced to adenocarcinoma (Törnell *et al.*, 1996).

EBNA-2 is a *trans*-activator of many cellular (*CD23*, *CD21*, *c-fgr*) and viral genes (*LMP-1*, *LMP-2*, *Cp*) but cannot bind to DNA directly. A cellular protein that mediates binding of *EBNA-2* to its response element was first described in the *LMP-2A* promoter (Zimber-Strobl *et al.*, 1993) and later shown also to have binding sites in the C and *LMP-1* promoters (Ling *et al.*, 1993a; Grossman *et al.*, 1994; Laux *et al.*, 1994b; Yalamanchili *et al.*, 1994). This protein turned out to be recombination signal binding protein Jκ (RBP-Jκ) (Grossman *et al.*, 1994; Henkel *et al.*, 1994; Waltzer *et al.*, 1994; Zimber-Strobl *et al.*, 1994), a ubiquitously expressed protein which is highly conserved in evolution, with homologues in species as distant as *Drosophila* (suppressor of hairless, *Su(H)*) (Furukawa *et al.*, 1992; Schweisguth & Posakony, 1992) and *Caenorhabditis elegans* (Christensen *et al.*, 1996). Its name is misleading since it proved not to bind to the immunoglobulin Jκ sequence (Tun *et al.*, 1994). The genetics of *Drosophila* provided a clue to its physiological function. RBP-Jκ/Su(H) is a DNA-binding protein and a downstream target of a cellular receptor called Notch (Fortini & Artavanis-Tsakonas, 1994), which in mammals is part of a family of related proteins. Ligand binding activates Notch, and subsequent processing and transport of Notch to the nucleus converts the repressor RBP-Jκ (Hsieh & Hayward, 1995; Waltzer *et al.*, 1995) into an activator and turns on target genes (Jarriault *et al.*, 1995; Hsieh *et al.*, 1996). EBNA-2 and activated Notch interact with similar regions in the RBP-Jκ molecule and fulfil similar functions (Hsieh *et al.*, 1996; Sakai & Honjo, 1997). EBNA-2 may therefore be regarded as a functional homologue of a constitutively active Notch receptor. By analogy with other viral oncoproteins, it seems likely that *EBNA-2* has functions additional to activation of RBP-Jκ. Binding of EBNA-2 to RBP-Jκ, although necessary, is not sufficient for *trans*-activation of EBNA-2-regulated promoters. In the *LMP-1* promoter, binding of PU.1 is additionally involved in *trans*-activation by EBNA-2 (Laux *et al.*, 1994b; Johannsen *et al.*, 1995), and other proteins involved are still to be identified. The region in EBNA-2 that interacts with RBP-Jκ has been mapped to amino acids 318–327 (Yalamanchili *et al.*, 1994; Ling & Hayward, 1995), a region highly conserved among the *EBNA-2* genes of EBV types 1 and 2 and HVP (Ling *et al.*, 1993b). It is one of the three regions of *EBNA-2*, including the C-terminal *trans*-activation domain and an N-terminal domain with unknown function, that are critically required for transformation (Cohen *et al.*, 1991). Additional details on the biochemistry and interaction with basal transcriptional machinery are given in Section 1.

In view of the functional equivalence between EBNA-2 and an activated Notch receptor, Notch signalling may partially substitute for EBNA-2 function in activation of viral and cellular genes in the EBNA-2-negative forms of latency.

4.1.5.4 *EBNA-3A, -3B and -3C*

EBNA-3A, *-3B* and *-3C* differ in type 1 and type 2 strains, but these differences do not affect the transforming ability of the viruses (Sample *et al.*, 1990; Tomkinson & Kieff, 1992b). *EBNA-3A* and *-C* are required for transformation, whereas *EBNA-3B* is not (Tomkinson & Kieff, 1992a; Tomkinson *et al.*, 1993). This is quite surprising, since *EBNA-3B* is a major target for recognition of latently infected cells by cytotoxic T cells (Moss *et al.*, 1996) and might be counterselected if it were not an essential gene for the viral life cycle *in vivo*. The EBNA-3C and -3B proteins have been reported to participate in transcriptional regulation of the *LMP-1*, *CD21* and *CD23*, and *CD40*, *CD77* and *vimentin* genes, respectively (Wang *et al.*, 1990a; Allday *et al.*, 1993; Silins & Sculley, 1994). EBNA-3A, -3B and -3C proteins were recently shown to inhibit transcriptional activation of EBNA-2-responsive promoters (Le Roux *et al.*, 1994) by preventing RBP-Jκ and EBNA-2/RBP-Jκ complexes from binding to their cognate RBP-Jκ binding sites (Waltzer *et al.*, 1996; Robertson *et al.*, 1996; Zhao *et al.*, 1996). EBNA-3 proteins are thus believed to counterbalance and finely tune the action of EBNA-2, and it is likely that they have other functions. EBNA-3C has been described as countering the action of the cyclin-dependent kinase inhibitor p16 INKLA and inactivating the retinoblastoma protein (Rb) functionally, like the viral proteins HPV E7 and adenovirus E1A (Parker *et al.*, 1996).

4.1.5.5 *LMP-1*

LMP-1 is the only known EBV gene which, acting alone, can transform rodent fibroblasts (Wang *et al.*, 1985). *LMP-1*-expressing rodent fibroblasts grow in low concentrations of serum, acquire anchorage-independent growth in soft agar and become tumorigenic. In primary B cells, *LMP-1* induces DNA synthesis and up-regulation of *CD23*, *CD21* and *CD54* (Peng & Lundgren, 1992).

LMP-1 is highly toxic if expressed at high levels (Hammerschmidt *et al.*, 1989; Floettmann *et al.*, 1996). When expressed at lower levels, it induces a number of phenotypic and functional changes such as up-regulation of adhesion molecules and activation markers (e.g. *CD23*, *CD39*, *CD40*, *CD54* and *CD58*; Wang *et al.*, 1988b, 1990b), restoration of immunological function, i.e. coordinated up-regulation of peptide transporters and HLA class-I and class-II molecules (Cuomo *et al.*, 1990; de Campos-Lima *et al.*, 1993b; Zhang *et al.*, 1994a; Rowe *et al.*, 1995) and induction of cyclin D2 (Arvanitakis *et al.*, 1995) and stress-response genes that prevent apoptosis, such as *bcl-2* and *A20* (Henderson *et al.*, 1991; Laherty *et al.*, 1992; Rowe *et al.*, 1994). Most of these functions are usually attributed to the ability of *LMP-1* to induce NFκB (Hammarskjold & Simurda, 1992), but this is not the only signalling pathway to which *LMP-1* contributes (Miller *et al.*, 1997). The molecular link between *LMP-1* and induction of the expression of genes such as *bcl-2* is in fact still missing (Martin *et al.*, 1993; Rowe *et al.*, 1994). When expressed in epithelial cells, LMP-1 inhibits cell differentiation in keratinocyte raft cultures *in vitro* (Dawson *et al.*, 1990) and induces morphological transformation and aberrant keratin expression (Fåhraeus *et al.*, 1990b) and severe epidermal hyperplasia in transgenic mice (Wilson *et al.*, 1990).

LMP-1 is absolutely required for both the initiation and maintenance of B-cell transformation by EBV (Kaye *et al.*, 1993). Genetic experiments have shown that the first transmembrane domain and the neighbouring amino acids of the cytoplasmic N-terminal domain are required for B-cell transformation (Kaye *et al.*, 1993), while the function of the 155 C-terminal amino acids can be complemented by co-cultivation of the cells on a fibroblast feeder layer (Kaye *et al.*, 1995). Constitutive *LMP-1* expression in EBV-transformed cells carrying a conditional *EBNA-2* gene has provided evidence that *LMP-1* in the absence of functional *EBNA-2* promotes survival of the cells without maintaining proliferation, similar to the stimulation of the endogenous CD40 receptor by CD40 ligand, which is even more effective in promoting cell survival than LMP-1 (Zimber-Strobl *et al.*, 1996). The functional similarity between LMP-1 and CD40 is corroborated by the fact that the two proteins recruit the same types of signalling molecules on the cytoplasmic side of the membrane. These molecules, which are known as tumour necrosis factor (TRAF) receptor-associated factors mediate activation of NFκB (Mosialos *et al.*, 1995). Deletion of the TRAF interaction domain within the LMP-1 molecule abolishes B-cell transformation by EBV (Izumi *et al.*, 1997).

4.1.5.6 *LMP-2A and -2B*

A clue to the function of LMP-2A has been provided by the observations that (i) it has eight tyrosine motifs that can be phosphorylated and interact with other kinases and adapter proteins; (ii) it is a substrate for members of the src family of kinases expressed in B cells (fyn, lyn) and is stably phosphorylated on tyrosine (Longnecker *et al.*, 1991; Burkhardt *et al.*, 1992); (iii) it is associated with another stably phosphorylated tyrosine kinase (syk) (Miller *et al.*, 1995a); (iv) it shares the sequence motif $(YXXL/I)_2$ with signal transducing subunits of antigen receptors in B and T cells (Reth, 1989; Beaufils *et al.*, 1993); and (v) it inhibits anti-immunoglobulin-mediated Ca^{2+} mobilization, PLCγ2 activation and anti-immunoglobulin-induced reactivation of the lytic cycle, which can be bypassed by TPA with Ca^{2+} ionophore (Miller *et al.*, 1994b, 1995a). These data are consistent with a model in which LMP-2A sequesters the receptor-associated tyrosine kinase, blocking its autophosphorylation and downstream signalling events (Miller *et al.*, 1995a).

Consistent with this model, *LMP-2A* and *-2B* proved not to be required for B-cell transformation by EBV *in vitro* (Longnecker *et al.*, 1992, 1993a,b), even though they contribute to the efficiency of transformation (Brielmeier *et al.*, 1996). Maintenance of latency and prevention of entry into the lytic cycle due to inhibition of the B-cell receptor may, however, be a more important issue for the virus *in vivo* than *in vitro*.

4.1.6 *Cellular genes induced during growth transformation by EBV*

Normal cells are induced to proliferate by signalling molecules (soluble molecules such as hormones and growth factors or matrix or cell-borne molecules) that interact with specific receptors, deliver a signal to the cell, transmit it into the nucleus and convert the cell to a new transcriptional programme and a proliferative response. As seen with oncogenes, malignant growth is a consequence of genetic changes which, on the one hand,

abrogate negative control mechanisms and, on the other, render the signalling process constitutive. This is achieved by shortcuts introduced into the signal transduction pathway at some level between the membrane and the nucleus.

Since different proliferative signals have to converge into a common programme leading to entry into S phase, the proteins that control these checkpoints, such as Rb and p53, are frequent targets for transforming viruses like papillomaviruses, SV40 and adenoviruses, which abrogate the cellular control mechanisms. In contrast, there is no evidence for a direct functional interaction between EBV latency proteins and either Rb or p53; however, it is likely that EBV indirectly affects these pathways. Recent data suggest that EBNA-3C overcomes the action of cyclin-dependent kinase inhibitor p16 and in this way affects cellular cycle control mechanisms (Parker *et al.*, 1996). In addition, *LMP-1* expression inhibits p53-mediated apoptosis (Okan *et al.*, 1995; Fries *et al.*, 1996).

In comparison with resting B cells, EBV-transformed lymphoblastoid cell lines show dramatic changes in their gene expression programme. In fact, EBV appears to mimic physiological B-cell activation brought about by antigen and helper T cells, and up-regulates many kinds of cell-surface molecules that might be important in signalling and growth regulation. Among the cellular genes induced by EBV are those that code for growth factors such as IL-5 (Paul *et al.*, 1990), IL-6 (Tanner & Tosato, 1992; Tanner *et al.*, 1996), IL-10 (Burdin *et al.*, 1993; Nakagomi *et al.*, 1994), TNFα and lymphotoxin (Estrov *et al.*, 1993; Gibbons *et al.*, 1994), thioredoxin (Wakasugi *et al.*, 1990), receptors such as the transferrin receptor, CD21, CD23, the TNFα receptor (Gibbons *et al.*, 1994; Wei *et al.*, 1994), TRAF-1 (Mosialos *et al.*, 1995), a protein related to the p40 subunit of the IL-12 receptor (Devergne *et al.*, 1996), G-protein-coupled receptors (Dobner *et al.*, 1992; Birkenbach *et al.*, 1993), activation markers (CD39), adhesion molecules (CD44, CD48, CD54, CD58) and molecules involved in cytoskeleton formation such as vimentin and actin-bundling protein (Birkenbach *et al.*, 1989; Mosialos *et al.*, 1994; Wang *et al.*, 1990b; Yokoyama *et al.*, 1991). It seems likely that these molecules participate in the induction and maintenance of growth transformation by EBV by establishing autocrine loops (only convincingly shown for lymphotoxin) or cell–cell-mediated cross-stimulatory pathways.

It should be borne in mind, however, that there is currently no way of testing such hypotheses. A conditional knock-out system for cellular genes (e.g. conditional induction of antisense RNA or ribozymes) is urgently required to elucidate the role of individual cellular genes for the maintenance of growth transformation.

4.2 Burkitt's lymphoma

4.2.1 *Molecular abnormalities in relation to the tumour-cell precursor*

4.2.1.1 *Translocation of the* c-myc *oncogene*

The discovery of non-random chromosomal translocations associated with Burkitt's lymphoma (Manolov & Manolova, 1972; Zech *et al.*, 1976; Bernheim *et al.*, 1981) paved the way to an understanding of the genetic derangements that are a central component of its pathogenesis.

From the observations that a chromosomal breakpoint on chromosome 8, band q24 is common to all three of the observed translocations in Burkitt's lymphoma and that the breakpoints are located on chromosomes 14, 2 and 22, at the heavy- and light-chain immunoglobulin loci (Croce *et al.*, 1979; Lenoir *et al.*, 1982; Malcolm *et al.*, 1982; McBride *et al.*, 1982), a strategy for identifying a potential oncogene on chromosome 8q24 was devised. Probes from the immunoglobulin regions were used to clone adjacent DNA fragments, which were soon shown to contain *c-myc* (Dalla-Favera *et al.*, 1982; Taub *et al.*, 1982; Adams *et al.*, 1983). Each of the chromosomal translocations results in the juxtaposition of *c-myc* to immunoglobulin sequences — either *c-myc* is translocated distal to heavy-chain sequences on chromosome 14q32, or light chain sequences from chromosomes 2p11 (kappa) or 22q12 (lambda) are translocated distal to *c-myc* on chromosome 8 (reviewed by Magrath, 1990; Lüscher & Eisenman, 1990). The *c-myc* gene is known to be important in the control of cell proliferation and cell death (Askew *et al.*, 1991; Evan *et al.*, 1992).

The juxtaposition of *c-myc* to immunoglobulin genes in a B-cell lymphoma suggests that the latter genes themselves have an important role to play in pathogenesis. Firstly, they are probably relevant to the genesis of the translocations, because recombinational events occur in immunoglobulin genes during B-cell ontogeny. The physiological breakage and relegation of the DNA strand to the regions in which the chromosomal breakpoints occur is unlikely to be due to chance; either the chromosomes are more readily broken in areas of the DNA strand that are exposed to recombinases, or translocations are actually created by the same recombinases that are responsible for VDJ rearrangements. Secondly, the transcriptional deregulation of the *c-myc* gene, which results from the translocation, is due to its juxtaposition to transcriptional control elements within the immunoglobulin locus (Madisen & Groudine, 1994; Polack *et al.*, 1993; Hörtnagel *et al.*, 1995). The net consequence of the translocation appears to be that *c-myc* is regulated as if it were an immunoglobulin gene, i.e. it is constitutively expressed in these immunoglobulin-synthesizing tumour cells. Thirdly, structural changes within the regulatory and coding regions of *c-myc*, reminiscent of somatic mutations in the hypervariable regions of the immunoglobulin genes, invariably accompany the translocation (Rabbitts *et al.*, 1984; Pelicci *et al.*, 1986; Magrath, 1990; Zajac-Kaye *et al.*, 1990; Bhatia *et al.*, 1995; Raffeld *et al.*, 1995).

Because the *c-myc*–immunoglobulin translocation is the central element in the pathogenesis of Burkitt's lymphoma, one possible role for cofactors is to increase the likelihood that a translocation will occur. On the basis of evidence derived by Southern blot that the J region of the immunoglobulin heavy chain locus involved in the translocation is not rearranged in a large fraction of Burkitt's lymphomas, it has been surmised that the translocation occurs early in B-cell differentiation (Bhatia *et al.*, 1995). This assumption is consistent with the notion that precursor B cells in the process of, or just about to, rearrange their immunoglobulin genes are particularly susceptible to translocation. By extrapolating from mouse models, it can be calculated that 10^{10} new B cells are generated daily in humans (Opstelten & Osmond, 1983). An environmental agent that increases the rate of B-cell ontogeny could simultaneously increase the frequency of occurrence of translocations, even if the latter is a purely stochastic event. Both EBV and malaria cause

B-cell hyperplasia and thus could influence the development of Burkitt's lymphoma in this way (Lenoir & Bornkamm, 1987). When both agents are present, as in African infants, the risk of developing a translocation is likely to be increased. Other agents, such as phorbol esters present in medicinal plants, have been proposed to increase the risk for chromosomal translocations additionally (Osato *et al.*, 1990; van den Bosch *et al.*, 1993).

EBV cannot only induce cell proliferation and thus increase on a statistical basis the likelihood that a *c-myc*–immunoglobulin translocation will arise, but it may also directly promote immunoglobulin gene recombination. Such gene recombination in lymphoid cells is dependent upon expression of the recombination activating genes *RAG-1* and *RAG-2*. To minimize the risk of aberrant recombination, the expression of these genes is limited to a narrow window in the development of B cells. EBV infection of sporadic Burkitt's lymphoma-negative cells and transfection of *EBNA-1* have been shown to up-regulate *RAG* expression (Kuhn-Hallek *et al.*, 1995; Srinivas & Sixbey, 1995). Expression of *LMP-1* and of *RAG-1* were, however, found to be mutually exclusive. The possibility that inappropriate *RAG* expression increases the likelihood of aberrant immunoglobulin gene recombination is supported by the observation that EBV-transformed, fetal liver-derived precursor B cells frequently develop translocations involving the immunoglobulin heavy-chain locus (Altiok *et al.*, 1989). B Lymphocytes that have undergone aberrant recombination are normally eliminated by apoptosis. Precursors to Burkitt's lymphoma cells with aberrant recombinations may be illegitimately rescued from apoptosis by EBV-encoded *LMP-1* if the malignant stage involves type II or type III latency phenotypes.

If the role of EBV in the pathogenesis of Burkitt's lymphoma were confined to the likelihood that a *myc* translocation occurs, it would follow that once such a translocation has developed the viral genome is no longer necessary.

4.2.1.2 *The Burkitt's lymphoma-cell phenotype resembles that of a germinal-centre cell*

While there is evidence that the chromosomal translocations occur in precursor B cells, the immunophenotype of Burkitt's lymphoma is that of a germinal-centre cell, indicating that differentiation must have occurred after the translocation. This may be an explanation for the essentially invariable expression of surface immunoglobulin, since differentiating B cells that fail to express a functional immunoglobulin molecule are destined to undergo apoptosis. The need for genetic changes, or possibly the expression of EBV genes that permit a translocation-bearing cell to avoid apoptosis, is likely to be an essential component of lymphomagenesis.

Consistent with the hypothesis that Burkitt's lymphoma cells have a germinal-centre phenotype is the presence of mutations in the hypervariable region of the heavy immunoglobulin chain in these cells (Chapman *et al.*, 1995; Klein *et al.*, 1995; Tamaru *et al.*, 1995), raising the possibility that exposure to antigens may be relevant to the pathogenesis of Burkitt's lymphoma. Burkitt's lymphoma cells do not, however, express activation antigens (Gregory *et al.*, 1987b) and in this respect, differ markedly from EBV-

transformed lymphoblastoid cell lines. This phenotype seems to be a consequence of the c-*myc* translocation (Polack *et al.*, 1996).

4.2.1.3 *Mutations in* p53 *in Burkitt's lymphoma*

Mutations in *p53* have been detected in up to 37% of biopsy samples from primary Burkitt's lymphomas (Gaidano *et al.*, 1991; Bhatia *et al.*, 1992). The incidence of *p53* mutation is independent of the geographic origin of the tumour, the chromosomal translocation or the association with EBV. The specific mutations in *p53* in Burkitt's lymphoma are distinct from those found in non-lymphoid tumours, as most are clustered in codons 213–248. The frequency of *p53* mutation is higher in cell lines, most of which are derived from recurrent tumours, more than 70% of which harbour mutations (Gaidano *et al.*, 1991; Farrell *et al.*, 1991; Bhatia *et al.*, 1992). This finding may indicate that *p53* mutation contributes to tumour progression. One consequence of the absence of *LMP-1* expression in Burkitt's lymphoma (Rowe *et al.*, 1987a) is the lack of its protective effects on *p53*-mediated apoptosis (Okan *et al.*, 1995; Fries *et al.*, 1996). Mutations in *p53* are rare in EBV-associated malignancies in which *LMP-1* is expressed (Spruck *et al.*, 1992; Sun *et al.*, 1992; Fåhraeus *et al.*, 1988).

4.2.2 *EBV infection in Burkitt's lymphoma*

Although chromosomal translocations affecting *c-myc* and the immunoglobulin loci are always present in Burkitt's lymphoma, EBV is not invariably found. About 95% of the cases in endemic areas of Africa and Papua–New Guinea contain EBV, but the prevalence in Burkitt's lymphoma outside the endemic areas varies with underlying disease and geographic region from 15 to more than 90% (see section 2.1). Both EBV type 1 and type 2 may be associated with Burkitt's lymphoma (Zimber *et al.*, 1986). A definitive conclusion about the pathogenetic role of EBV in Burkitt's lymphoma cannot be reached solely on the basis of epidemiological observations; molecular studies provide a number of additional insights and are the most likely source of unequivocal evidence for a pathogenetic role of the virus.

4.2.2.1 *EBV is monoclonal in Burkitt's lymphoma*

One requirement that must be fulfilled if EBV is to subserve a pathogenetic role in Burkitt's lymphoma is that the virus must not only be present in the tumour cells but must also have entered the cell before its final conversion to a malignant lymphoma. Since malignant tumours originate from a single cell which contains all of the necessary genetic abnormalities, EBV should be present in all tumour cells and should also be monoclonal, i.e. all tumour cells should be derived from the same EBV-infected cell. The detection of a homogeneous number of terminal repeats in EBV episomes in Burkitt's lymphoma tumour cells showed that the virus is indeed clonal and indicated that the tumour developed from a single EBV progenitor cell (Raab-Traub & Flynn, 1986; Neri *et al.*, 1991).

4.2.2.2 *Integration of viral DNA in Burkitt's lymphoma cells*

Many oncogenic viruses contribute to tumorigenesis by insertional mutagenesis, e.g. by activation of proto-oncogenes and/or inactivation of tumour suppressor genes. Integration of the EBV genome has been observed in several Burkitt's lymphoma-derived cell lines (Henderson *et al.*, 1983; Delecluse *et al.*, 1993a); however, no preferential integration site has, so far, been identified. Moreover, integration is not a *sine qua non*, since EBV-positive Burkitt's lymphoma cells may (rarely) lose the viral genomes when grown *in vitro* (Takada *et al.*, 1995), and EBV-positive and EBV-negative cells co-exist in a Burkitt lymphoma line (Trivedi *et al.*, 1995).

4.2.2.3 *Expression of EBV genes in EBV-associated Burkitt's lymphoma*

If EBV exerts a direct oncogenic effect, one or more viral genes should be expressed in tumour cells. This criterion is also fulfilled, although the pattern of expression of EBV proteins is markedly different in Burkitt's lymphoma and in lymphoblastoid cell lines. This suggests that there are basic differences in the mechanisms whereby EBV transforms B cells on the one hand and contributes to the development, or maintenance, of Burkitt's lymphoma on the other. Although the full range of latency genes is expressed in EBV-transformed cell lines, only *EBNA-1* and the *EBERs* are invariably expressed in Burkitt's lymphoma cells (Rowe *et al.*, 1986, 1987a; Niedobitek *et al.*, 1995). Rare cells within a tumour may express other EBV genes, such as *EBNA-2*, *LMP* and *BZLF1* (Niedobitek *et al.*, 1995), and may even enter the lytic cycle (Gutiérrez *et al.*, 1993).

The differences in latent gene expression in lymphoblastoid cell lines and Burkitt's lymphoma are regulated at the level of promoters in these cell types. In lymphoblastoid cell lines, all of the EBNA proteins are encoded from spliced transcripts derived from a common long precursor initiated from promoters in the W and/or C *Bam*HI fragments of the EBV genome, whereas in Burkitt's lymphoma *EBNA-1* is transcribed from the Q promoter (Schaefer *et al.*, 1995). Q Promoter-initiated transcripts that encode *EBNA-1* are negatively regulated by *EBNA-1* (Sample *et al.*, 1992).

A recent study revealed that transgenic mice that express *EBNA-1* under the control of immunoglobulin enhancer develop B-cell lymphomas (Wilson *et al.*, 1996c). This suggests that *EBNA-1* may have additional properties beyond maintenance and regulation of the viral genome and that it contributes to the development of malignancy.

4.2.2.4 *Expression only of EBNA-1 is associated with reduced immunogenicity*

The limited expression of EBV gene products in Burkitt's lymphoma cells provides significant protection from immune recognition, as most of the CTL response is directed against EBNA-3 proteins. In addition, EBNA-1 is not processed or presented in class I MHC molecules (Levitskaya *et al.*, 1995). The EBNA-1 protein contains a simple repeat of glycine and alanine. Transfer of this repeat element to a heterologous protein sequesters that protein from processing and expression within MHC class I. This unique property enables an EBV-infected cell that expresses only *EBNA-1* to escape immune recognition.

Burkitt's lymphoma cells not only lack EBV-derived immunogenic proteins, they also express low levels of HLA class-I molecules (Masucci *et al.*, 1987; Andersson *et al.*, 1991; Gavioli *et al.*, 1992; Imreh *et al.*, 1995), adhesion molecules, including LFA-1 (CD11a), ICAM-1 (CD54) and LFA-3 (CD58), and co-stimulatory molecules, B7-1 and B7-2 (CD80 and CD86), that participate in antigen presentation, while CD10, CD38 and CD77 are un-regulated (Gregory *et al.*, 1988, 1990; Billaud *et al.*, 1990; Ling *et al.*, 1989; Magrath, 1990; Martel-Renoir *et al.*, 1995). As a consequence, Burkitt's lymphoma cells are virtually invisible to the immune system, as evidenced by the fact that the cells are unable to elicit an allostimulatory T-cell response (Cuomo *et al.*, 1990). The phenotype of Burkitt's lymphoma cells is a consequence of high *c-myc* expression and non-expression of the viral proteins involved in growth promotion, except for EBNA-1 (Polack *et al.*, 1996).

4.2.2.5 *The proliferation programme driven by c-myc–immunoglobulin is incompatible with expression of EBNA-2 and LMP-1 in the type-III latency programme*

After the discovery of the chromosomal translocations in Burkitt's lymphoma cells, it was suspected that, in analogy to the well-documented cooperation of oncogenes (Hunter, 1991), EBV viral gene products would cooperate with an activated *c-myc* gene to promote a fully malignant phenotype. It was therefore not anticipated that *EBNA-2* and *LMP-1* would have adverse effects on the proliferation of Burkitt's lymphoma cells and even abrogate their tumorigenic potential (Torsteinsdottir *et al.*, 1989; Cuomo *et al.*, 1992; Floettmann *et al.*, 1996). This can be explained, at least in part, by the fact that *EBNA-2* inhibits the transcriptional regulatory elements of the immunoglobulin heavy chain locus. *EBNA-2* down-regulates IgM expression, and, in cells in which *c-myc* transcription is driven by translocation into the immunoglobulin heavy-chain locus, it down-regulates *c-myc* expression concomitantly (Jochner *et al.*, 1996).

Thus, while *EBNA-2* is absolutely required for proliferation of EBV-infected normal cells, switching on the function of *EBNA-2* inhibits proliferation of Burkitt's lymphoma cells with the t(8;14) translocation (Kempkes *et al.*, 1996). In cells with the variant t(2;8) translocation, *EBNA-2* does not affect *c-myc* expression directly but slows proliferation in an indirect manner (Polack *et al.*, 1996). *EBNA-2* expression and proliferation mediated by constitutive activation of c-*myc* are thus mutually exclusive.

EBV-positive Burkitt's lymphoma cells tend to change their phenotype and switch to the type III latency programme, expressing *EBNA-2* and *LMP-1*, when explanted into tissue cultures, which at first sight seems contradictory to what is stated above. Burkitt's lymphoma cells in type I latency are highly susceptible to apoptosis and evolve variants resistant to apoptosis *in vitro* which have switched to the type III latency programme (Gregory *et al.*, 1991). Under these conditions, however, the cells proliferate significantly more slowly (Falk *et al.*, 1993) and express lower levels of *c-myc* RNA and protein than Burkitt's lymphoma cells in type-I latency (Jochner *et al.*, 1996), due to the effects of *EBNA-2* on heavy-chain-regulated *c-myc* (Bornkamm *et al.*, 1988; Spencer &

Groudine, 1991), while the expression of *EBNA-2* and *LMP-1* seems sufficiently high to protect the cells from apoptosis.

4.2.2.6 *Contribution of the viral strategy for latent persistence to lymphomagenesis*

The different type I latency programmes together represent a strategy necessary for the virus to maintain a permanent reservoir of viral genomes in B cells in normal EBV-infected individuals. This strategy also contributes to lymphomagenesis (see section 4.2.1 above; Rowe *et al*, 1992; Chen *et al.*, 1995a; Miyashita *et al.*, 1995). Under physiological conditions, the type-I latency programme allows maintenance of EBV DNA without risk of elimination by CTL, as discussed elsewhere. This programme, which under physiological conditions appears to operate only in non-proliferating B cells, is maintained in Burkitt's lymphoma cells.

4.2.2.7 *EBNA-1 subtypes*

Recently, subtypes of EBNA-1 that differ in their amino-acid sequence have been identified (Bhatia *et al.*, 1996). These differences do not seem to reflect geographical regions. Interestingly, a subtype marked by alanine at position 487 is the commonest form in normal individuals but is rarely found in tumours. In contrast, the subtype marked by leucine at 487 but containing several additional amino-acid substitutions is frequently found in Burkitt's lymphomas and not in normal individuals (Gutiérrez *et al.*, 1997). This subtype, *V-leu*, differs only at position 487 from another subtype, *V-pro*, which is present in normal lymphocytes but only in association with other variants. These data suggest that the subtypes arise *in vivo* and that mutations in *EBNA-1* may be relevant to the pathogenesis of Burkitt's lymphoma.

4.2.3 *Effects of malaria on B-cell activation and EBV infection*

While epidemiological evidence for a role of malaria as a predisposing factor (see section 2.1.4.1) is strong, it will be important to identify the specific mechanism whereby malaria influences the pathogenesis of Burkitt's lymphoma. To date, the mechanism has not been identified, although experimental evidence derived from studies in mice provide paradigms that may be applicable to Burkitt's lymphoma.

In a study conducted by Jerusalem (1968), 64% of mice infected with *Plasmodium berghei* eventually developed malignant lymphoma, while Osmond *et al.* (1990) showed that infection of mice with *P. bergei yeollii* increased the proportion of immature B cells (pro-B cells) in the bone marrow and decreased the pool of mature B cells. It is this expansion of precursor B cells that is likely to be responsible for the increased susceptibility of *Plasmodium*-infected mice to the development of lymphoma.

In a study of concurrent infection, 10 of 12 adult mice infected with *P. berghei yeollii* and Moloney leukaemia virus developed lymphoma, in comparison with one of 11 infected only with Moloney virus and none of 10 infected with malaria alone (Wedderburn, 1970). Similar findings were reported by Salaman *et al.* (1969). Moloney virus does not contain an oncogene and activates oncogene expression by a process of promoter insertion. The combination of precursor B-cell hyperplasia and genetic lesions

induced by Moloney virus is likely to account for the increased frequency of lymphomas in the doubly infected mice.

A similar situation may apply to African children. If malarial infection in humans also expands pro-B- and pre-B-cell populations, the risk for developing Burkitt's lymphoma may be increased, as the pool of cells that could develop a *c-myc*–immunoglobulin translocation is larger. EBV infection may well add an additional potentially tumorigenic lesion, although the molecular pathways involved are unlikely to include promoter insertion.

While expansion of precursor B-cell populations in children in regions where malaria is hyperendemic has not been examined, there is evidence that the B-cell system is hyperactive during the course of infection. Children with malaria have very high serum levels of IgG and IgM, most of which are not due to anti-plasmodial antibodies and plateau after the age of five to six years (McGregor, 1970). Turnover of IgG is as much as seven times greater in adults from holoendemic malarious regions than among those from control regions (Cohen *et al.*, 1961; Cohen & McGregor, 1963).

Chronic malaria, like HIV infection, leads to a shift in the helper T-cell response towards T_h2 cells (von der Weid & Langhorne, 1993). T_h2 cytokines, such as IL-10, suppress the humoral arm of the immune response, support the early steps of B-cell immortalization by EBV (Burdin *et al.*, 1993) and suppress CTL function. As a consequence, the number of B lymphocytes latently infected with EBV increases, while the ability of T cells to suppress the outgrowth of EBV-infected lymphoblastoid cells is impaired (Gunapala *et al.*, 1990; Moss *et al.*, 1983a; Whittle *et al.*, 1984; Lam *et al.*, 1991).

In addition, EBV seroconversion is known to occur much earlier in populations of low socioeconomic status or those who live in crowded conditions than in populations in which families are smaller and have more spacious accommodation (Niederman *et al.*, 1968; Hinuma *et al.*, 1969; Henle & Henle, 1970, 1979). In developing countries, seroconversion occurs in the majority of children by the age of three years (Diehl *et al.*, 1969; Kafuko *et al.*, 1972), occurring earlier in rural areas than in urban zones (Biggar *et al.*, 1981), and is usually not associated with a clinical syndrome (Diehl *et al.*, 1969). It is possible, as originally suggested by de Thé (1977), that early infection with EBV predisposes to the development of EBV-associated Burkitt's lymphoma because of differences in the immune system of infants and older individuals and, in the context of African children, because of the presence of coincident B-cell hyperplasia and immunosuppression caused by malaria.

4.2.4 *Plant products*

Phorbol esters interact with protein kinase C and are potent inducers of cell differentiation. They can induce the differentiation of Burkitt's lymphoma-derived cell lines (Benjamin *et al.*, 1984; Ho *et al.*, 1987; Osato *et al.*, 1990). Several authors have demonstrated that extracts of *Euphorbia tirucalli* and related plants, or even extracts of soil or reservoir water near places where *Euphorbia* plants are growing, can induce the expression of EA and VCA in Raji cells (Ito *et al.*, 1981). Lin *et al.* (1982) showed that

TPA, a phorbol ester, increases viral replication, as measured by an increase in genome copies. This potential activation of EBV replication could result in an increased number of lymphocytes that become infected with EBV.

Similar extracts have been reported to increase the outgrowth of EBV-transformed B cell lines and to decrease EBV-specific T-cell killing (Osato *et al.*, 1990; Imai *et al.*, 1994b). In addition, of potentially considerable interest, Osato *et al.* (1990) observed that dual exposure of B lymphocytes to EBV and deoxyphorbol ester, the active principle of *Euphorbia*, increased the numbers of chromosomal translocations and deletions involving chromosome 8. Thus, the presence of these tumour-promoting compounds in the environment could influence both EBV infection and the genesis of chromosomal abnormality.

4.2.5 *Genetic disposition*

Although familial Burkitt's lymphoma has been reported (see section 2.1.2), genes that could account for an increased risk for the development of Burkitt's lymphoma have not been identified, although it is probable that genetic determinants of the response to relevant cofactors such as malaria, e.g. the presence of sickle-cell trait (Williams, 1966; Pike *et al.*, 1970; Nkrumah & Perkins, 1976), may influence the risk for development of this tumour in Africa. Similarly, inherited immunodeficiency syndromes, such as X-linked lymphoproliferative disorder (Provisor *et al.*, 1975; Purtilo, 1976), are associated with a markedly increased risk for the development of non-Hodgkin's lymphomas (Harrington *et al.*, 1987), which sometimes contain *c-myc*–immunoglobulin gene translocations and have the histological appearance of Burkitt's lymphoma (Egeler *et al.*, 1992).

4.2.6 *Burkitt's lymphoma in AIDS patients*

Burkitt's lymphoma arising in the context of AIDS is associated with EBV infection in only 30–40% of cases, which is greater than that observed in sporadic Burkitt's lymphoma (Ernberg & Altiok, 1989; Gaidano & Dalla-Favera, 1995). This percentage is, however, much lower than that in other non-Hodgkin's lymphomas (see section 4.3.1.3).

In comparison with other non-Hodgkin's lymphomas, AIDS-associated Burkitt's lymphoma develops earlier in the progress of the disease and in less severely immunocompromised individuals. While the characteristics of endemic Burkitt's lymphoma may also apply to AIDS-related disease, there are some important differences: (i) EBV is found in only a proportion of AIDS-related Burkitt's lymphomas, (ii) the patients are much older than those with endemic Burkitt's lymphoma, and (iii) the pattern of *c-myc* translocation is different, involving the switch region rather than the VDJ region.

The clinical, molecular and viral features of AIDS-related Burkitt's lymphoma indicate that its pathogenesis is similar to that of sporadic Burkitt's lymphoma in immunocompetent individuals; however, in the context of AIDS, these lymphomas occur at increased incidence. This suggests the contribution of an AIDS-related factor. That this factor is unlikely to be simply immunosuppression is indicated by the fact that Burkitt's lymphomas occur only rarely in transplant patients and then only in association with rela-

tively low immunosuppression (Niedobitek *et al.*, 1997b). Chronic immune stimulation by malaria has been suggested to play a role in the pathogenesis of endemic Burkitt's lymphoma, and stimulation by e.g. schistosomiasis may be involved in the development of cases in other regions (Araujo *et al.*, 1996). Indeed, it has been proposed that HIV-induced polyclonal B-cell proliferation may contribute to the development of AIDS-related Burkitt's lymphoma (Kalter *et al.*, 1985; Seigneurin *et al.*, 1987; Gaidano & Dalla-Favera, 1995).

For a discussion of lymphomas in AIDS patients, see section 4.3.1.3.

4.3 Other Non-Hodgkin's lymphomas and lymphoproliferative conditions

4.3.1 *Immunosuppressed patients*

The most conspicuous features of human infections with EBV are their ubiquitous distribution in the adult population and that they are mostly asymptomatic. Equally impressive is the high frequency of EBV-positive lymphomas occurring in patients with severe immunosuppression caused by e.g. inherited immune defects and iatrogenic or HIV-associated immunosuppression. These facts together serve as the strongest evidence for a direct oncogenic effect of EBV infection in B cells, when physiological immune regulation and natural defence barriers have broken down.

4.3.1.1 *Primary immune defects due to genetic abnormalities with EBV-positive lymphoproliferation as one consequence*

Patients with X-linked lymphoproliferative syndrome suffer from a number of serious complications after primary infection with EBV. The genetic basis of this syndrome is described in Section 1 (Skare *et al.*, 1993). Other relevant primary immune defects that may involve EBV-related complications are Wiskott-Aldrich syndrome, common variable immunodeficiency and ataxia telangectasia. These genetic disorders result in different types of defects affecting the immune system, in particular T-cell or NK-cell maturation, but also B-cell maturation in common variable immunodeficiency (Purtilo, 1991; Sander *et al.*, 1992; Nakanishi *et al.*, 1993). Lymphoproliferative disorders in these patients encompass a disease spectrum ranging from polyclonal lymphoid hyperplasia at one end to overtly malignant monoclonal high-grade non-Hodgkin's lymphoma at the other (for a review, see Niedobitek *et al.*, 1997c). In particular, in the Wiskott-Aldrich and X-linked lymphoproliferative syndromes, many of the lymphoproliferations are polyclonal (Falk *et al.*, 1990; Nakanishi *et al.*, 1993). Most cases of non-Hodgkin's lymphoma arising in association with primary immune defects are of the B-cell phenotype and are EBV-positive (Falk *et al.*, 1990; Purtilo, 1991; Sander *et al.*, 1992; Nakanishi *et al.*, 1993). Whereas the pattern of EBV gene expression in most polyclonal lymphoproliferations is similar to that in lymphoblastoid cell lines (type-III latency), monoclonal lesions often display a type-I latency. In some cases, EBNAs have been expressed in the absence of detectable LMP-1. Whether this represents a new form of EBV latency or merely reflects technical problems is currently uncertain. The recent detection of a similar phenotype in EBV-associated smooth-muscle tumours (Lee *et al.*,

1995b) suggests that the EBNA-positive/LMP-1 phenotype indeed represents a novel form of stable EBV latency.

Efforts are being made to clone the *XLP* gene and to characterize its function (see Section 1). This will eventually afford a better understanding of the role of EBV in the development of lymphoproliferation in these patients. In general terms, the frequent occurrence of virus-associated lymphoproliferation in patients with genetic immune defects illustrates the significance of intact immune regulation in maintaining the EBV–host balance. Notably, however, immunosuppression does not appear to be associated with nasopharyngeal carcinoma, an epithelial tumour. The range of lymphoproliferations observed may reflect progression from polyclonal lymphoblastoid cell line-like lesions to monoclonal lymphomas as one of the pathogenetic mechanisms of EBV-positive non-Hodgkin's lymphomas (see discussion on post-transplant lymphoproliferative disorders below). In situations with type II or type III latency in the lymphoproliferative tissue, it is conceivable that EBV contributes to driving cell proliferation or, at least, contributes to extended cell survival.

4.3.1.2 *Post-transplant lymphoproliferative disorders*

Post-transplantation lymphoproliferative disorders are a major complication in allograft recipients, occurring in 1–20% of patients (Nalesnik & Starzl, 1994). The incidence tends to be lowest for renal transplant recipients and highest for lung transplant patients (Nalesnik & Startzl, 1994; Montone *et al.*, 1996a; Swinnen, 1996), which may reflect more intensive use of immunosuppressive therapy in the latter. The incidence of post-transplant lymphoproliferative disorders in bone-marrow transplant patients is generally low, although it may be up to 24% in recipients of mismatched T cell-depleted marrow (Shapiro *et al.*, 1988). More recently, similar lesions have been reported in patients being treated for rheumatoid disease with methotrexate (Kamel *et al.*, 1993, 1994). The lymphoproliferations in these patients appear to be morphologically and biologically similar to post-transplant lymphoproliferative disorders and are thus not discussed separately in this context. The vast majority of such cases have been shown to be of B-cell origin and to be associated with EBV infection (Locker & Nalesnik, 1989; Swerdlow, 1992; Craig *et al.*, 1993); however, EBV-negative non-Hodgkin's lymphomas of B- and T-cell phenotypes occur occasionally in transplant recipients (Nalesnik & Starzl, 1994). These may represent sporadic lymphomas developing independently of iatrogenic immunosuppression, and are not discussed here.

The general consensus is that post-transplant lymphoproliferative disorders developing in recipients of solid-organ transplants are of host origin, while those occurring in bone-marrow transplant patients are of donor origin (Zutter *et al.*, 1988; Chadburn *et al.*, 1995a; Weissmann *et al.*, 1995); however, exceptions in both directions have been reported. Thus, Zutter *et al.* (1988) demonstrated that two of 12 cases of post-transplant lymphoproliferative disorder arising in bone-marrow recipients were of host origin, in keeping with the reported persistence of EBV-positive B cells of host origin in bone-marrow transplant patients (Gerhartz *et al.*, 1988). More surprisingly perhaps, Larson

et al. (1996) and Mentzer *et al.* (1996) showed recently that post-transplant lymphoproliferative disorders of donor origin may develop in recipients of solid organs.

For reasons as yet unknown, post-transplant lymphoproliferative disorder frequently develops in the gastrointestinal tract and in the transplanted organ (Guettier *et al.*, 1992; Opelz & Henderson, 1993; Palazzo *et al.*, 1993; Mentzer *et al.*, 1996; Randhawa *et al.*, 1996). Histologically, these conditions represent a spectrum ranging from benign polyclonal, polymorphic lymphoproliferations at one end to frankly malignant monoclonal, monomorphic lymphomas at the other (Swerdlow, 1992; Craig *et al.*, 1993). Typically, B cells in these lymphoproliferations express a broad spectrum of virus-encoded latent proteins, including EBNA-1, EBNA-2 and LMP-1 (Young *et al.*, 1989a; Thomas *et al.*, 1990). This form of EBV latency (type III) is similar to that seen in lymphoblastoid cell lines *in vitro* and, accordingly, these cells usually display a lymphoblastoid-like pattern of cellular gene expression, including lymphocyte activation and adhesion molecules (Thomas *et al.*, 1990). A large proportion of cases of post-transplant lymphoproliferative disorder also show lytic infection, usually in a small subset of cells (Patton *et al.*, 1990; Rea *et al.*, 1994; Montone *et al.*, 1996b). Whether this is sufficient justification for the inclusion of acyclovir in the treatment of these conditions, as often advocated, is uncertain (Zutter *et al.*, 1988; Patton *et al.*, 1990; Davis *et al.*, 1995). It seems likely that entry into the lytic cycle is simply a reflection of the underlying immune defect.

It is generally held that the above-mentioned morphological spectrum of post-transplant lymphoproliferative disorder represents an evolutionary process (Craig *et al.*, 1993). Thus, it is believed that the pathogenesis of the condition starts with EBV-driven polyclonal B-cell proliferation; the acquisition of additional genetic changes then leads to the emergence of dominant B-cell clones and eventually to fully developed malignant lymphomas which are morphologically indistinguishable from lymphomas arising in immunocompetent individuals. This scheme is based on the demonstration in studies of immunoglobulin gene rearrangement and analysis of the structure of the viral terminal repeat sequences that some polymorphic cases are polyclonal. Histologically monomorphic large-cell lymphomas, however, have been shown to be monoclonal using both approaches. In addition, in one group of polymorphic lesions minor clonal components are detectable on the background of polyclonal B-cell proliferations (Locker & Nalesnik, 1989). Furthermore, only monomorphic, monoclonal cases of post-transplant lymphoproliferative disorder contain alterations involving oncogenes or tumour suppressor genes, e.g. *c-myc* translocations or mutations of the N-*ras* and *p53* genes (Delecluse *et al.*,1995b; Knowles *et al.*, 1995). In a recent study, 24 separate lesions in one patient were all clonally distinct (Chadburn *et al.*, 1995b). In a series of 11 patients with recurrent post-transplant lymphoproliferative disorders, the recurrent lesions were either relapses of the original disease or clonally distinct. In some cases, progression from polymorphic disease to frankly malignant lymphoma, including Hodgkin's disease and T-cell lymphomas, was observed. Significantly, no primarily monomorphic case recurred as polymorphic disease, further supporting the notion that the disease progresses from early polymorphic polyclonal lesions to monomorphic monoclonal disease (Wu *et al.*, 1996).

This concept of the pathogenesis of post-transplant lymphoproliferative disorder is also supported by the frequent regression of lesions in response to a reduction in immu-

nosuppressive therapy (Starzl *et al.*, 1984). The evidence described above would imply that early polyclonal lesions are more susceptible to this approach than late monoclonal lesions. Indeed, it has been observed that cases that develop less than one year after transplantation have a much higher response rate than those occurring more than one year later (Armitage *et al.*, 1991). Similarly, polyclonal cases appear to respond better than monoclonal lesions; however, this distinction is not absolute, and a trial of reduced immunosuppression has been recommended even in patients with monoclonal disease (Nalesnik *et al.*, 1992). Lucas *et al.* (1996) demonstrated recently that the frequency of EBV-specific CTL precursors in the peripheral blood of bone-marrow recipients returned to normal in nine of 13 patients within six months after transplantation. In this series, one patient with low precursor CTL levels four months after transplantation developed post-transplant lymphoproliferative disorder, further underlining the significance of suppressed T-cell immunity in its pathogenesis. Regression of lymphoproliferative lesions has also been reported in patients with rheumatoid disease after withdrawal of methotrexate therapy, further reflecting the similarity of some of these lesions to post-transplant lymphoproliferative disorder (Kamel *et al.*, 1993; Salloum *et al.*, 1996; Thomason *et al.*, 1996).

Studies to identify transplant patients who are at risk of developing post-transplant lymphoproliferative disorder have defined certain risk factors. Individuals who are EBV-negative before transplantation and undergo seroconversion after transplantation appear to be particularly prone to development of the condition (Ho *et al.*, 1985; Walker *et al.*, 1995). Further risk factors are the duration and severity of immunosuppression, including use of OKT3, CDw52 and anti-LFA1 antibodies (Opelz & Henderson, 1993; Nalesnik & Starzl, 1994; Gerritsen *et al.*, 1996; Walker *et al.*, 1995). More direct approaches to monitoring the risks of individual patients include quantitative PCR assays to measure the viral DNA load in peripheral blood and techniques for counting the number of virus-carrying B cells in blood or allograft biopsy samples (Randhawa *et al.*, 1992; Crompton *et al.*, 1994; Riddler *et al.*, 1994; Savoie *et al.*, 1994; Kenagy *et al.*, 1995).

Several recent studies have indicated a certain heterogeneity of post-transplant lymphoproliferative disorders. Yoshizawa *et al.* (1993) isolated a cell line from a case which resolved in response to reduced immunosuppression. This cell line, SUBL, displayed a pre-B-cell phenotype and genotype and carried a t(2;3)(p11;q27) translocation, indicating that factors other than proliferation of mature B cells can also give rise to the condition. Furthermore, some EBV-positive T-cell lymphomas have been reported (Borisch *et al.*, 1992b; Kumar *et al.*, 1993; Waller *et al.*, 1993) in which the virus was found by in-situ hybridization to be present in only rare neoplastic cells; thus, the role of EBV in the pathogenesis of these tumours remains uncertain (Borisch *et al.*, 1992b). The virus is consistently detected in the rare cases of Hodgkin's disease that occur in the context of transplantation (Garnier *et al.*, 1996), reflecting the detection of EBV in most cases of HIV-associated Hodgkin's disease (Uccini *et al.*, 1990; Boiocchi *et al.*, 1993b; Herndier *et al.*, 1993).

The analysis of EBV gene expression patterns in post-transplant lymphoproliferative disorders shows considerable variability both between and within lesions. Thus, not all cases display type-III latency (Rea *et al.*, 1994; Delecluse *et al.*, 1995a; Oudejans *et al.*,

1995a). Particularly in monomorphic lymphomas, EBV latent protein expression may be restricted to type-I or type-II latency (Rea *et al.*, 1994; Delecluse *et al.*, 1995a). Moreover, even in those cases in which both EBNA-2 and LMP-1 (indicative of type-III latency) can be identified, expression of these proteins is often seen in variable subsets of cells (Thomas *et al.*, 1990; Rea *et al.*, 1994; Delecluse *et al.*, 1995a; Oudejans *et al.*, 1995b). Thus, a relatively small proportion of cells may display type-III latency and others show type-I or type-II latency. In addition, cells in which EBNA-2 is expressed in the absence of LMP-1 have been observed, as also seen in infectious mononucleosis (Oudejans *et al.*, 1995b; Niedobitek *et al.*, 1997b).

4.3.1.3 *AIDS*

In the general population, the incidence of non-Hodgkin's lymphomas is increasing rapidly, but they still account for only about 4% of all cancers (Parkin *et al.*, 1992). In contrast, non-Hodgkin's lymphomas are very common in HIV-infected individuals, primarily at extranodal sites. Particularly common are primary central nervous system lymphomas, which account for 1.6% of non-Hodgkin's lymphomas in the HIV-negative population (Krogh-Jensen *et al.*, 1994). In a recent Norwegian study, the results of 153 autopsies of AIDS patients were evaluated, representing 73% of all AIDS patients who died. The overall accumulated cancer incidence was 35%. In this series, more cases of primary central nervous system lymphoma (19 cases) than systemic non-Hodgkin's lymphoma (12 cases) were identified (Goplen *et al.*, 1997). In a study in the United States, central nervous system lymphomas constituted only some 20% of all AIDS-related non-Hodgkin's lymphomas (Beral *et al.*, 1991). In larger surveys, 1–4% of AIDS patients had lymphomas (for review, see IARC, 1996). These figures are likely to be underestimates because of the usually low autopsy rates for AIDS patients. Morphologically, AIDS-related non-Hodgkin's lymphomas fall into two broad groups: diffuse large B-cell non-Hodgkin's lymphomas, which often show a prominent immunoblastic component, and Burkitt's lymphoma and Burkitt's-like lymphoma. As lymphomas in AIDS patients have been reviewed recently (IARC, 1996), we focus here only on the EBV-related lymphomas and mechanistic aspects.

(*a*) *Viral factors*

The two histopathological types of AIDS-related non-Hodgkin's lymphoma show striking differences in their relationship to EBV, suggesting different pathogenetic mechanisms. Most diffuse large B-cell non-Hodgkin's lymphomas, particularly those with immunoblastic differentiation, and all AIDS-related central nervous system lymphomas are EBV-positive (MacMahon *et al.*, 1991; for review, see IARC, 1996). Diffuse large B-cell lymphomas have been reported to occur relatively late in AIDS patients (Gaidano & Dalla-Favera, 1995), and more advanced depression of the immune system is a risk factor for their development (Pedersen *et al.*, 1991). The exceedingly rare primary effusion lymphomas fall into the group of diffuse large B-cell non-Hodgkin's lymphomas on morphological grounds; however, they appear to be a special case because of the unusual site of presentation and because the tumour cells often harbour KSHV/HHV8 in addition to EBV (Cesarman *et al.*, 1995). This suggests that the two

viruses cooperate in the pathogenesis of this disease, but the nature of the co-infection is at present uncertain. More recently, primary effusion lymphomas harbouring KSHV/-HHV8 only have also been described (Chang *et al.*, 1994), and thus the role of EBV in the development of this special type of non-Hodgkin's lymphoma remains uncertain.

Regardless of histological type, most AIDS-related non-Hodgkin's lymphomas appear to be monoclonal, both with respect to their antigen receptor genes and to the EBV episomes (Ballerini *et al.*, 1993; Delecluse *et al.*, 1993c; Shibata *et al.*, 1993). This situation differs from that in lymphoproliferative processes occurring in transplant patients (see section 4.3.1.2), which are frequently polyclonal; however, Delecluse *et al.* (1993c) demonstrated that rare cases may be polyclonal and thus resemble post-transplant lymphoproliferative disorder.

(*b*) *Disturbances of immunity as cofactors*

As EBV-positive, AIDS-related B-cell lymphomas consistently lack the HIV genome, a direct contribution of HIV to tumorigenesis beyond suppression of the immune system is unlikely (Knowles, 1993; IARC, 1996). Shiramizu *et al.* (1994) reported rare cases of HIV-containing tumours, but this work requires confirmation.

As discussed below, HIV may contribute by inducing severe immunosuppression, leading to a loss of EBV-specific T-cell immunity, or by chronic stimulation of the B-cell system. The relative risk for AIDS-related non-Hodgkin's lymphoma increases with duration of HIV infection (Rabkin *et al.*, 1992) and to a certain extent with immune suppression (Muñoz *et al.*, 1993). T Cell-mediated immunosurveillance is known to play an important part in controlling the proliferation of EBV-infected B lymphocytes in humans (for review, see Rickinson *et al.*, 1992; Masucci & Ernberg, 1994). The spontaneous outgrowth of EBV-positive cells in peripheral blood from AIDS patients is greater than that in immunocompetent individuals (Birx *et al.*, 1986; Ragona *et al.*, 1986; Rinaldo *et al.*, 1986). Similarly, the number of EBV-carrying lymphocytes detected in peripheral lymphoid tissues is greater in HIV-infected individuals than in non-immunocompromised controls (Niedobitek *et al.*, 1992a). This evidence indicates that disturbances in EBV-specific immunity are a pathogenetic factor in the development of some EBV-associated, AIDS-related non-Hodgkin's lymphomas. The location to the central nervous system of one predominant group of AIDS-associated lymphomas may reflect the fact that this site is less accessible for immunosurveillance (MacMahon *et al.*, 1991).

The absence of those viral latent genes that are commonly recognized by cytotoxic T cells from AIDS-associated Burkitt's lymphomas is in keeping with the generally better immune system of these patients in comparison with those with EBV-associated diffuse large-cell non-Hodgkin's lymphomas. It also suggests that the pathogenetic mechanisms leading to the development of these two lymphoma types are different (Knowles, 1996); however, a few AIDS-related cases of non-Hodgkin's lymphoma fall between the two morphological categories, diffuse large B-cell non-Hodgkin's lymphoma and Burkitt's lymphoma, and these cases carry *c-myc* translocations and may thus represent evolution of Burkitt's lymphoma *in vivo* (Delecluse *et al.*, 1993c). This process may be similar to the shift of Burkitt's lymphoma cells to the lymphoblastoid cell line-like phenotype observed *in vitro*. That this may also occur *in vivo* is suggested by the detection of iso-

lated cells expressing LMP-1 and/or EBNA-2 in cases of endemic and AIDS-related Burkitt's lymphoma (Hamilton-Dutoit *et al.*, 1993a; Niedobitek *et al.*, 1995). Such a shift could be expected to coincide with progressive deterioration of the immune system.

(*c*) *Oncogenes and genetic abnormalities as possible cofactors*

There is some evidence that disruption of the cytokine network contributes to the pathogenesis of AIDS-related non-Hodgkin's lymphoma. Thus, it has been shown that B cells from HIV-infected patients, unlike those of normal controls, constitutively express IL-6 and TNFα (for review see Gaidano & Dalla-Favera, 1995). This is of relevance, as IL-6 is known to promote the growth of EBV-positive B cells (Tanner & Tosato, 1992). IL-6 expression has been shown in endothelial cells and macrophages admixed with the neoplastic cells in biopsy samples of AIDS-related non-Hodgkin's lymphomas (Emilie *et al.*, 1992a). Moreover, EBV-positive AIDS-related Burkitt's lymphomas have been shown to express IL-10 (Emilie *et al.*, 1992b; Masood *et al.*, 1995), another cytokine which may directly or indirectly promote the growth of EBV-positive B cells (Vieira *et al.*, 1991; Herbst *et al.*, 1996b). It appears therefore, that IL-6 and IL-10 (and possibly other cytokines) can contribute to the development of AIDS-related non-Hodgkin's lymphoma through paracrine or autocrine regulatory loops.

As discussed previously, AIDS-related Burkitt's lymphomas, both EBV-positive and EBV-negative, have been consistently shown to harbour the characteristic *c-myc* translocation. These translocations have been detected in a minority of diffuse large B-cell lymphomas and cases with morphological features between large B-cell lymphoma and Burkitt's lymphoma (Ballerini *et al.*, 1993; Delecluse *et al.*, 1993c; Bhatia *et al.*, 1994). Although *p53* point mutations have been detected in 60% of Burkitt's lymphomas in AIDS patients, they were not seen in any other type of non-Hodgkin's lymphoma (for review, see IARC, 1996). Chromosomal translocations in AIDS-related non-Hodgkin's lymphomas frequently involve *bcl-6*, a proto-oncogene that affects B-cell maturation, which maps to 3q27 (Ye *et al.*, 1993). This is the region mainly affected in large-cell lymphomas, rearrangements being seen in 20% of cases. Although the rearrangements of *bcl-6* and *c-myc* occur in approximately 20% of cases of diffuse large B-cell non-Hodgkin's lymphoma, they have been reported to be mutually exclusive (Gaidano *et al.*, 1994).

Point mutations were detected in N-*ras* and K-*ras* in four of 27 AIDS-related non-Hodgkin's lymphomas investigated, but not in non-Hodgkin's lymphomas in immunocompetent hosts (Ballerini *et al.*, 1993).

Deletion of the long arm of chromosome 6 at band q27 was described in five of 13 large-cell lymphomas, some of which were EBV-positive (Pastore *et al.*, 1996). This rearrangement is seen in a wide range of non-Hodgkin's lymphomas in immunocompetent hosts (Gaidano *et al.*, 1992). Genetic abnormalities in chromosome 1q have been reported in AIDS-related cases of Burkitt's lymphoma by several groups, similar to those seen in endemic cases (Bernheim & Berger, 1988; Polito *et al.*, 1995), site 1q 21-25 being frequently involved.

Thus, AIDS-related non-Hodgkin's lymphomas, whether EBV-associated or not, frequently show genetic changes implicated in the pathogenesis of malignant lymphomas, including *c-myc* translocations, *p53*, N-*ras* and K-*ras* point mutations and deletions in the long arm of chromosome 6 (Gaidano & Dalla-Favera, 1995).

(*d*) *Pathogenesis of EBV-associated, AIDS-related non-Hodgkin's lymphoma: A scenario*

AIDS-related non-Hodgkin's lymphomas thus appear to fall into two broad categories with distinct pathogenetic pathways. Diffuse large B-cell lymphomas occur in severely immunocompromised individuals, are mostly EBV-positive and express type-II or -III latency patterns. While most of these cases are monoclonal and morphologically malignant, rare EBV-positive polyclonal lymphoproliferations have been described. It appears, therefore, that the pathogenesis of these lesions is similar to that of post-transplant lymphoproliferative disorder (Gaidano & Dalla-Favera, 1995), with an initial EBV-driven proliferation of B cells leading eventually to the outgrowth of fully malignant lymphomas. This process would depend on the acquisition of additional genetic change, e.g. *c-myc* or *bcl-6* rearrangements (Gaidano & Dalla-Favera, 1995).

The clinical, molecular and viral features of AIDS-related Burkitt's lymphoma suggest a pathogenesis similar to that of sporadic Burkitt's lymphoma. The comparatively high incidence of these lymphomas in AIDS patients points to the contribution of an AIDS-related factor independent of HIV-mediated immunosuppression. As these lymphomas have been shown to be HIV-negative (Knowles, 1993), a direct role of HIV is unlikely. The primary event in AIDS-related Burkitt's lymphoma appears to be chronic stimulation of the immune system, leading to polyclonal B-cell hyperplasia; this process may well be driven initially by HIV infection. The relatively low rate of EBV infection in these lymphomas suggests that the function of the virus can be substituted by other, as yet unidentified, factors.

4.3.2 *T-Cell lymphomas*

The relatively frequent detection of EBV in T-cell lymphomas does not fit easily into the well-established B-cell lymphotropism of the virus *in vitro*. As noted earlier (see Section 1), a subset of T cells has been shown to express CD21 or a related molecule that could act as the receptor for EBV. Interpretation of the detection of EBV in T-cell non-Hodgkin's lymphomas and an assessment of the role of the virus in the pathogenesis of T-cell lymphomas are complicated by two factors. Firstly, if EBV infection of certain T cells *in vitro* leads to a predominantly lytic infection, EBV infection of T cells may be accidental rather than part of the viral strategy to establish persistent infection. Such accidental infection of a cell type not adapted to latent EBV infection may contribute to the development of EBV-associated T-cell lymphomas. Secondly, in many cases, the virus is detected in only a small proportion of tumour cells (Anagnostopoulos *et al.*, 1996). Particularly in T-cell lymphomas, this problem is compounded by the polymorphism of the tumour cells. Thus, it may be difficult to decide if an individual cell is a tumour cell or a reactive lymphoid cell. Furthermore, the identification of EBV in neoplastic cells usually rests on the detection of the EBERs, which may not be expressed

even in the presence of EBV. Nevertheless, there seems to be good evidence to suggest that the reported detection of EBV in a fraction of cells in T-cell lymphomas reflects the actual situation (Asada *et al.*, 1994; Imai *et al.*, 1996).

The question of the significance of the virus in the pathogenesis of these lymphomas thus arises. Although the virus is present at the onset of the neoplastic process, it may subsequently be lost from the tumour cells. While there is now some evidence to suggest that this may happen *in vitro* (Takada *et al.*, 1995), it has not yet been shown to occur *in vivo*. The alternative scenario would be secondary infection of established neoplastic T cells with the virus. While this would exclude a contribution of the virus to the initial neoplastic process, it would be compatible with a contribution to the disease process, similar to the role of late *p53* gene mutations in the progression of follicular lymphomas. The frequent expression of the LMP-1 protein of EBV in T-cell lymphomas (d'Amore *et al.*, 1996) would seem to argue in favour of such a role.

EBV is often monoclonal in T-cell lymphomas containing a subset of virus-carrying tumour cells, which argues against simple superinfection of an established tumour (Ott *et al.*, 1992). Studies of EBV-associated T-cell lymphomas at primary presentation and at relapse could be informative.

In addition to the peripheral T-cell lymphomas occurring in western populations, in which subpopulations of the tumour cells often carry the virus, there are clearly some T-cell non-Hodgkin's lymphomas in which the presence of monoclonal EBV genomes is a defining characteristic of the neoplastic population. This group is exemplified by angiocentric sinonasal T-cell lymphomas, which occur predominantly in Asian populations. The role of the virus in the pathogenesis of these tumours is uncertain; however, the clonal nature of the EBV genome and its detection in virtually all tumour cells suggests a role of EBV early in tumorigenesis. While mechanisms have not been identified in lymphomatous granulomatosis, it has been shown that the EBV-infected B cells express LMP-1 (Takeshita *et al.*, 1996).

4.4 Hodgkin's disease

Over the last decade, evidence has accumulated implicating EBV in the pathogenesis of Hodgkin's disease. Up to 50% of cases in western countries (Weiss *et al.*, 1989a; Herbst *et al.*, 1991a; Armstrong *et al.*, 1992) and up to 100% in some other populations (Weinreb *et al.*, 1996b) carry the virus in the presumably neoplastic HRS cells. Sero-epidemiological studies have shown elevated antibody titres to EBV antigens in Hodgkin's disease patients both at the time of presentation and some years before the onset of the disease (Mueller *et al.*, 1989; Mueller, 1997). Furthermore, patients with infectious mononucleosis have a fourfold increase in risk for developing Hodgkin's disease (Muñoz *et al.*, 1978). More direct evidence linking EBV with Hodgkin's disease has accumulated (see also section 1.2.2). Thus, EBV DNA has been detected in tumour biopsies and has been localized to malignant HRS cells by in-situ hybridization (Weiss *et al.*, 1987; Anagnostopoulos *et al.*, 1989; Weiss *et al.*, 1989a). Significantly, it has been shown by EBER in-situ hybridization that the virus is present in virtually all tumour cells in EBV-associated Hodgkin's disease (Wu *et al.*, 1990; Herbst *et al.*, 1992). This finding

is consistent with the detection of monoclonal EBV episomes in cases of Hodgkin's disease by Southern blot hybridization and indicates that the EBV infection took place before initiation of clonal cellular proliferation (Weiss *et al.*, 1987; Anagnostopoulos *et al.*, 1989). In agreement with this idea, it has been shown that patients with multifocal Hodgkin's disease have the virus in all affected tissues and that the virus persists in recurrent disease (Coates *et al.*, 1991a; Boiocchi *et al.*, 1993a; Brousset *et al.*, 1994; Vasef *et al.*, 1995).

Further evidence suggesting a role for EBV in the pathogenesis of Hodgkin's disease comes from the analysis of EBV gene expression in HRS cells. Transcriptional studies by RT-PCR have shown that *EBNA-1* expression in Hodgkin's disease tissues is driven exclusively by the F promoter; *LMP-1* and usually *LMP-2A* and *LMP-2B* are also expressed, in addition to *EBER*s (Deacon *et al.*, 1993). Immunohistochemical studies have now confirmed that EBNA-1, LMP-1 and LMP-2A are indeed expressed by the HRS cells in EBV-associated Hodgkin's disease, while EBNA-2 is consistently not detectable (Herbst *et al.*, 1991a; Pallesen *et al.*, 1991a; Delsol *et al.*, 1992; Grässer *et al.*, 1994; Niedobitek *et al.*, 1997a). This pattern of EBV latent gene expression is consistent with type-II latency. These studies indicate that the viral genome is actively expressed in HRS cells and thus reveal that EBV is not merely a silent passenger in these cells.

The main function of *EBNA-1* is maintenance of the viral episome (Yates *et al.*, 1984); however, a recent study of *EBNA-1*-transgenic mice suggests that this gene may also contribute to the development of lymphoid tumours (Wilson *et al.*, 1996a). Whether *EBNA-1* expression in HRS cells contributes to the pathogenesis of Hodgkin's disease remains uncertain.

LMP-2A expression is thought to block the induction of the lytic viral cycle after cross-linking of the B-cell receptor complex, by acting as a dominant negative inhibitor of Src and Syk kinases (Longnecker & Miller, 1996). In line with this scheme and with the frequent detection of LMP-2A in HRS cells (Niedobitek *et al.*, 1997c), it has been shown that EBV lytic cycle proteins are expressed only infrequently in HRS cells (Pallesen *et al.*, 1991c; Brousset *et al.*, 1993). Several studies suggest that *LMP-2A* and *LMP-2B* are not required for EBV-induced B-cell transformation (Longnecker *et al.*, 1992, 1993a,b); however, recent work by Brielmeier *et al.* (1996) indicates that *LMP-2A* enhances the efficiency of transformation and that this effect is not dependent on prevention of entry into the lytic cycle. Thus, *LMP-2A* expression may contribute to the malignant phenotype of HRS cells in EBV-associated Hodgkin's disease.

Of particular relevance is the detection of LMP-1 in HRS cells in virtually all cases of virus-associated Hodgkin's disease (Herbst *et al.*, 1991a; Pallesen *et al.*, 1991a; Delsol *et al.*, 1992) and the ability of LMP-1 to induce expression of activation markers such as CD23, CD30 and CD70 (Wang *et al.*, 1988b, 1990a; Isaacson *et al.*, 1992; Hamilton-Dutoit *et al.*, 1993a); however, these antigens are strongly expressed in HRS cells of both EBV-positive and EBV-negative cases.

There is growing evidence to suggest that EBV infection modulates the phenotype of Hodgkin's disease. Thus, EBV is preferentially associated with the development of mixed cellularity Hodgkin's disease. Moreover, the presence of EBV in HRS cells has

been shown to correlate with increased expression of lymphocyte activation antigens and decreased expression of the CD20 B-cell antigen (Bai *et al.*, 1994). Of particular relevance is the association of EBV infection with the expression of T_h2 cytokines such as IL-10 and IL-6 in HRS cells (Herbst *et al.*, 1996b; Klein *et al.*, 1996). Thus, although definite proof of an etiological role of EBV in the pathogenesis of Hodgkin's disease has yet to be attained, the available evidence strongly implicates the virus as a cofactor in the pathogenesis and morphogenesis of a significant proportion of cases.

It has been observed that cases of Hodgkin's disease arising in the setting of iatrogenic or HIV-induced immunosuppression are almost invariably EBV-associated. Moreover, regression of EBV-positive cases of Hodgkin's disease has been reported after restoration of the immune system (Berger & Delecluse, 1993), providing further evidence for an etiological role of EBV in the pathogenesis of Hodgkin's disease.

4.5 Nasopharyngeal carcinoma

4.5.1 *EBV infection*

4.5.1.1 *Molecular and biochemical studies*

EBV-positive nasopharyngeal carcinoma cells cannot yet be cultivated *in vitro*, and only a small proportion of tumour samples can be propagated in nude mice. Therefore, EBV infection in nasopharyngeal carcinoma has been characterized primarily by analysis of viral nucleic acids and proteins in samples obtained at biopsy and tumours passaged in nude mice (Raab-Traub *et al.*, 1983; Busson *et al.*, 1988). EBV episomes have been detected in nasopharyngeal carcinoma samples by electron microscopy (Kaschka-Dierich *et al.*, 1976) and by restriction enzyme analyses based on identification of the ends of the EBV genome (Raab-Traub & Flynn, 1986). Clonal EBV episomes are detected in such samples (Raab-Traub & Flynn, 1986), indicating that every copy of the viral genome within each cell is identical and the EBV episomes in every cell within the tumour are identical. The detection of homogeneous EBV molecules indicates that the tumour cells are a clonal expansion of a single cell that was infected with EBV.

In addition, in some tumour samples, particularly from endemic areas, faint ladder arrays of fragments representing linear genomes are also detected. This suggests that in occasional cells within the tumour the virus may be reactivated from latent infection. It is likely that this sporadic replication is the source of the antigenic stimulus that provokes the elevated antibody response to viral replicative antigens.

As episomal EBV DNA is present in multiple copies in nasopharyngeal carcinoma, it is difficult to determine whether EBV is also integrated; however, evidence of EBV integration was detected by pulse field analysis in four of 17 samples of nasopharyngeal carcinoma (Kripalani-Joshi & Law, 1994). Although integration may promote changes in cellular or viral expression that could contribute to tumour development, it is probably not a necessary event for development of this tumour.

4.5.1.2 *EBV expression*

EBV expression in nasopharyngeal carcinoma is different in several striking respects from that detected in transformed lymphocytes. The first studies of viral expression in samples of nasopharyngeal carcinomas revealed that sequences from the *Eco*RI-*Dhet* and the *Bam*HI K fragments were consistently transcribed (Raab-Traub *et al.*, 1983). These sequences were subsequently shown to encode the LMPs and EBNA-1, respectively, Surprisingly, the sequences that encode EBNA-2, a viral protein essential for transformation of lymphocytes, are not transcribed in nasopharyngeal carcinoma.

The pattern of gene expression suggested by transcriptional studies has for the most part been confirmed by the identification of specific viral proteins with monoclonal antibodies. LMP-1 and EBNA-1 have been detected, while EBNA-2, -3A, -3B, -3C and -LP are usually not found (Fåhraeus *et al.*, 1988; Young *et al.*, 1988). LMP-1 is not always detected in all cells or in all tumours on immunoblots, and EBNA-2 has not been identified in tumour samples, possibly due to a lack of suitable reagents (Stewart & Arrand, 1993). The BZLF1 protein has been detected in a few cells in some nasopharyngeal carcinomas, and spliced *BZLF1* mRNA and other mRNAs encoding lytic cytoproteins can also be detected by RT-PCR (Cochet *et al.*, 1993; Martel-Renoir *et al.*, 1995).

As the mRNAs that encode EBNAs all initiate at the W or C promoter in lymphocytes, it was of interest to determine the structure of the *EBNA-1* mRNA in nasopharyngeal carcinomas. Intriguingly, it was shown to initiate from sequences within *Bam*HI Q at a previously unidentified promoter, Q (Smith *et al.*, 1993; Tsai *et al.*, 1995). The Q promoter is complex and is apparently both autoregulated and governed by interferon regulatory factors (Sample *et al.*, 1992; Sung *et al.*, 1994).

In nasopharyngeal carcinoma, two different mRNAs encode LMP-1: the well-characterized, 2.8-kb *LMP-1* mRNA and a second, 3.7-kb mRNA (Gilligan *et al.*, 1990a). The 3.7-kb mRNA initiates from a promoter within the terminal repeat unit, which is transactivated *in vitro* by the SP1 transcription factor (Sadler & Raab-Traub, 1995b). Consistent transcription of *LMP-2* has been detected in nasopharyngeal carcinomas by northern blot analysis and RT-PCR (Busson *et al.*, 1992a). One study suggested that the *LMP-2B* or *TP2* transcript is preferentially expressed in nasopharyngeal carcinoma in comparison with matched B-cell lines (Smith & Griffin, 1991). Antibodies to LMP-2 were found highly specifically in patients with nasopharyngeal carcinoma (Frech *et al.*, 1993; Lennette *et al.*, 1995), suggesting that this protein is expressed in the tumour *in vivo*. In nasopharyngeal carcinoma, *EBNA-2*, *-3A*, *-3B*, *-3C* and *-LP* are not expressed, yet the promoters for *LMP-1* and *LMP-2* are apparently active, as the genes are transcribed. It is presently unclear how expression of these genes is regulated in the absence of the EBNA-2 and EBNA-3 proteins. Several studies have shown that methylation of the viral genome is an important regulatory element. The viral genome in nasopharyngeal carcinoma cells is extensively methylated, with the exception of *oriP* and regulatory elements for the *LMP-1* and *LMP-2* promoter (Ernberg *et al.*, 1989; Minarovits, 1991, 1994b; Hu *et al.*, 1991b). It is particularly striking that although the coding sequences for *LMP-1* are methylated, the promoter region is methylated only in those tumours in which the LMP-1 protein is not detected.

In addition to these RNAs, which are known to encode protein, a family of intricately spliced mRNAs was originally identified in samples of nasopharyngeal carcinomas (Hitt *et al.*, 1989; Gilligan *et al.*, 1990b, 1991). Sequence analysis of cDNA revealed that the RNAs are 3′ co-terminal but differentially spliced (Smith *et al.*, 1993; Sadler & Raab-Traub, 1995a). The various RNAs contain different exons, forming novel ORFs at least one of which has been shown to encode a protein (Gilligan *et al.*, 1991; Fries *et al.*, 1997) that has been detected in most nasopharyngeal carcinoma samples. Although these RNAs are most abundant in nasopharyngeal carcinoma, they have also been identified in transformed lymphocytes and in Burkitt's lymphoma (Brooks *et al.*, 1993a).

The most abundant RNAs in EBV-infected cells are small nuclear RNAs transcribed by RNA polymerase III (Lerner *et al.*, 1981; Arrand & Rymo, 1982). These *EBERs* are present at approximately 10^6 copies per cell but are not necessary for lymphocyte transformation (Swaminathan *et al.*, 1991). They are, however, expressed in all of the malignancies associated with EBV and presumably contribute in some way to the maintenance of latency *in vivo* (Wu *et al.*, 1991). Interestingly, expression of the *EBERs* seems to be down-regulated during differentiation. Thus, samples of nasopharyngeal carcinoma that show differing degrees of differentiation lack EBER expression in differentiated areas (Pathmanathan *et al.*, 1995a). Extensive screening showed the presence of EBERs in 4 of 5 (80%) subtype 1, 71 of 73 (97%) subtype 2 and 71 of 73 (97%) subtype 3 tumours. In the subtype 1, EBER expression was largely confined to the basal area and was not detected in regions of differentiation (Tsai *et al.*, 1996b). The EBERs are also not detected in the permissive EBV infection, hairy leukoplakia (Gilligan *et al.*, 1990b).

These findings indicate that nasopharyngeal carcinoma represents a latent infection with EBV involving consistent expression of specific viral genes in a pattern distinct from that found in transformed B lymphocytes. The properties of the viral proteins expressed in nasopharyngeal carcinoma are reviewed in sections 1 and 4.1 This unique state of latency is also found in several other malignancies linked to EBV, including Hodgkin's disease and T-cell lymphoma, and has been called type II latency (Rickinson & Kieff, 1996; see Section 1).

4.5.1.3 *Phenotype and cellular gene expression*

The presence of an intense lymphoid stroma is a characteristic feature of undifferentiated carcinomas of the nasopharyngeal type; the functional role of this stroma has been a matter of debate. Recent studies have suggested that in undifferentiated carcinomas the stroma is required to support tumour cell growth (Busson *et al.*, 1987; Agathanggelou *et al.*, 1995). The difficulty in establishing nasopharyngeal carcinoma xenografts in nude mice may reflect loss of the infiltrating stroma (Busson *et al.*, 1987).

Expression of many markers, including potential factor receptors, usually expressed in B cells has been described in primary nasopharyngeal carcinoma samples. These include CD23, CD24, Ia antigen and Cdw70 (Ebbers *et al.*, 1985; Billaud *et al.*, 1989; Rousselet & Tursz, 1992; Niedobitek *et al.*, 1992c; Karran *et al.*, 1995). The expression of adhesion molecules is also altered, with elevated expression of ICAM-1 (CD54) and reduced expression of LFA3 (CD58) in comparison with EBV-immortalized B cells

(Busson *et al.*, 1992b). These effects are probably due to expression of *LMP-1* in nasopharyngeal carcinoma cells.

Interestingly, clinical analysis of nasopharyngeal carcinomas with and without LMP-1 expression suggested that the LMP-positive tumours grew more rapidly but that the LMP-1-negative tumours were more likely to recur and had an increased tendency to metastasize (Hu *et al.*, 1995). This difference may be due to the fact that B7 (CD80/CD87) is expressed in LMP-1-positive but not in LMP-1-negative tumours (Agathanggelou *et al.*, 1995).

4.5.1.4 *EBV infection and transformation of epithelial cells* in vitro

Although EBV readily infects and transforms B lymphocytes, human epithelial cells cannot easily be infected or transformed; however, monkey epithelial cells were transformed by transfer of specific EBV DNA fragments cloned into cosmid vectors (Griffin & Karran, 1984). The fragments that had transforming ability included the *Bam*HI A region of the genome which has since been shown to be expressed in nasopharyngeal carcinomas (Hitt *et al.*, 1989; Gilligan *et al.*, 1990b). Some attempts to infect human epithelial cells directly have resulted in morphological transformation but not permanent transformation or EBNA expression (Desgranges & de Thé, 1977; Huang *et al.*, 1978a). In another study of epithelial cells infected *in vitro*, evidence of EBV replication was detected only in wild-type strains obtained directly from throat washings, suggesting distinct biological properties of some strains (Sixbey *et al.*, 1983).

The difficulty of infecting epithelial cells may be due to differences in expression of the EBV receptor, CD21 or CR2. Evidence for expression of the receptor is conflicting, depending on the monoclonal antibody used and the source of epithelium (Young *et al.*, 1989c; Sixbey *et al.*, 1987). Transfer of CD21 into an epithelial cell line facilitated infection, and a high level of infected cells was detected early after infection with the Akata strain of EBV (Li *et al.*, 1992). The genome was rapidly lost from these cells, and some cells spontaneously entered the replicative cycle; however, cell clones could be established that retained the EBV genome in episomal form and expressed *EBNA-1* and *LMP-1* (Knox *et al.*, 1996). The stably infected clones seemed to be impaired in their ability to differentiate or to allow viral replication. Clones that lacked EBV were also impaired in differentiation, suggesting that impairment in differentiation influences the ability of EBV to establish a latent, transforming infection.

The mode of viral entry may also influence the outcome of infection. It has been shown that secretory IgA facilitates the entry of EBV into epithelial cells (Sixbey & Yao, 1992). The cellular organization also modulates EBV infection, such that EBV is transcytosed by EBV-specific IgA directly through the epithelium in polarized cultures, but EBV replicative gene products are detected in unpolarized cultures (Gan *et al.*, 1997).

In some epithelial cell lines, LMP-1 has been reported to inhibit differentiation and cause morphological transformation, with decreased cytokeratin expression (Dawson *et al.*, 1990; Fåhraeus *et al.*, 1990a; Niedobitek *et al.*, 1992d). Decreased expression of E-cadherin and increased invasive ability has also been described in epithelial lines expressing LMP-1 (Fåhraeus *et al.*, 1992). In transgenic mice in which LMP-1 was

expressed in skin, epithelial hyperplasia and altered expression of keratin 6 were observed (Wilson *et al.*, 1990).

LMP-1 expression induces expression of the epidermal growth factor receptor, specifically in epithelial cells, and elevated levels of epidermal growth factor receptor are also detected in nasopharyngeal carcinoma (Zheng *et al.*, 1994c; Miller *et al.*, 1995b). As discussed in section 4.1, two domains have been identified in the carboxy terminus of *LMP-1* that can activate NFκB (Huen *et al.*, 1995). A recent study indicated that the *TRAF* interacting domain is responsible for induction of expression of the epidermal growth factor receptor (Miller *et al.*, 1997). This suggests that *LMP-1* activates two distinct signalling pathways: one that activates NFκB and a second pathway mediated by *TRAF* activation that induces expression of genes like *EGFR* and also activates NFκB.

Several EBV replicative proteins have been shown to have different effects in epithelial cells and lymphoid cells or to have transforming properties in rodent cell lines. Although only *BZLF1* can disrupt latency in lymphoid cell lines, in infected epithelial cell lines such as NPC-KT and D98/HR1, the immediate early gene *BRFL1* can also disrupt latency (Zalani *et al.*, 1996). In addition, *BZLF1* has been shown to interact with *p53* (Zhang *et al.*, 1994b), the p65 component of NFκB (Gutsch *et al.*, 1994), and induce expression of TGFβ. The EBV early gene, *BHRF1*, is homologous to the *bcl2* gene and can block apoptosis induced by various agents (Henderson *et al.*, 1993). *BHRF1* can also complement transformation by the adenovirus *E1A* gene (Theodorakis *et al.*, 1996). Another early gene encoded by the *BARF1* ORF can transform rodent fibroblasts and induce tumorigenicity in the EBV-negative Louckes Burkitt's lymphoma cell line (Wei *et al.*, 1994). As expression of EBV replicative gene products occurs in only a few percent of nasopharyngeal carcinoma cells *in vivo*, it is unlikely, however, that they contribute to malignant growth.

4.5.1.5 *Detection of EBV infection in normal, premalignant and malignant nasopharyngeal tissues*

The site and state of infection in normal nasopharynx and in populations at high risk for developing nasopharyngeal carcinoma have been investigated in several studies. In patients with infectious mononucleosis, EBV DNA and mRNA encoding EA were detected in sloughed epithelial cells (Lemon *et al.*, 1978; Sixbey *et al.*, 1984). This suggested that the nasopharyngeal epithelial cells were the source of the infectious virus that is detected in saliva during infectious mononucleosis. Evidence of EBV replication has also been detected in epithelial cells in parotid tissue, where high copy numbers of EBV genomes were detected by in-situ hybridization (Wolf *et al.*, 1984). A recent study also showed evidence of EBV replication in epithelial cells adjacent to an EBV-positive T-cell lymphoma (Wen *et al.*, 1997). The epithelial cells had high copy numbers of EBV DNA and were BZLF1-positive but EBER-negative, while the malignant lymphocytes were positive for EBER and LMP-1 expression. In contrast, studies of normal nasopharyngeal mucosa have shown evidence of EBV only in lymphocytes in which EBER-positive and BZLF1-positive cells were occasionally detected (Tao *et al.*, 1995). EBV-infected lymphocytes were detected in nasal polyps, and some were positive for BZLF1

expression (Tao *et al.*, 1996). Similarly, Karajannis *et al.* (1997) detected isolated EBV-positive B cells but no EBV-positive epithelial cells in throat washings from patients with infectious mononucleosis, suggesting that lymphocytes are the source of virus secreted into the oropharynx in reactivated infection.

Comprehensive screening of a large Chinese population resulted in the identification of 1267 individuals with IgA to VCA. Of the 203 from whom a biopsy sample was taken, 46 had early nasopharyngeal carcinoma and an additional 12 had detectable nasopharyngeal carcinoma within 12 months. EBV was detected by Southern blot hybridization in 14 samples from individuals without detectable nasopharyngeal carcinoma (Desgranges *et al.*, 1982), but it was not determined whether the tissues were normal or whether the patients had occult nasopharyngeal carcinoma.

Attempts to culture transformed or EBV-infected cells taken at biopsy resulted in the establishment of EBV-positive lymphoid lines from nasopharyngeal tissues from patients with and without nasopharyngeal carcinoma and from adenoidal tissue from other patients; however, epithelial cell lines were not established (de Thé *et al.*, 1970; Takimoto *et al.*, 1989). Further, attempts led to the development of cell lines in which EBV genomes were initially detected but were lost over time (Gu *et al.*, 1983; Lin *et al.*, 1993b). It is unclear whether these cell lines represent the tumour cells.

EBV markers for EBNA or latent or replicative genes were not detected in samples of normal tissue from patients without histological evidence of nasopharyngeal carcinoma, and were found only in samples with nests of tumour cells (Huang *et al.*, 1978b; Sam *et al.*, 1993). Subsequent screening showed EBV in all biopsy samples of nasopharyngeal carcinoma, but only the genome was detected in a subset of cells from carcinoma *in situ* (Yeung *et al.*, 1993). Samples from carcinomas *in situ* with microinvasion had a higher proportion of EBV-positive cells. An extensive screening of 5326 biopsy samples from the ear-nose-and-throat clinic at the University of Malaya resulted in the detection of nasopharyngeal carcinoma in 1744 samples; 56 samples had carcinoma *in situ* and invasive carcinoma, and only 11 samples showed dysplasia or carcinoma *in situ* without adjacent invasive carcinoma. In these samples, EBER and LMP-1 were detected in all cells, and analysis of the EBV termini revealed clonal EBV (Pathmanathan *et al.*, 1995a,b).

Some studies have suggested that EBV is detected only in discrete areas within a nasopharyngeal carcinoma, while in other studies homogeneous infection is detected throughout the samples (Wu *et al.*, 1991; Lin *et al.*, 1994; Pathmanathan *et al.*, 1995a,b). This variation may reflect differences in the method of detection or the state of infection. EBV DNA or EBER expression has been looked for as an indicator of the presence of EBV in many studies, but EBV DNA may not be detected in cells with a very low copy number and EBER expression may vary, as EBERs are not expressed in the permissive infection, hairy leukoplakia, or within areas of differentiation in tumours of mixed histological type (Gilligan *et al.*, 1990a; Pathmanathan *et al.*, 1995a,b). [The Working Group noted that the role of EBV in primary infection of epithelial cells is controversial.]

Taken together, these studies indicate that EBV infection of epithelial cells is rare. It may occur during primary infection but, in normal asymptomatic infection, EBV is

harboured in lymphoid cells. The rare event that leads to the development of nasopharyngeal carcinoma may enable establishment of a latent EBV infection in epithelium. The changes that predispose the cells to this event may have already induced changes in growth properties, which were originally suggested by Lenoir and de Thé (1978). The impaired differentiation observed by Knox *et al.* (1996) may have a correlate *in vivo* in the dysplasia described by Yeung *et al.* (1993). Furthermore, the study of Pathmanathan *et al.* (1995b) suggests that the combination of EBV oncogene expression and pre-existing cellular changes results in the rapid proliferation of an infected epithelial cell, which rapidly develops into malignant, invasive carcinoma (Raab-Traub, 1992a,b).

4.5.1.6 *Strain variation*

Many of the cancers associated with EBV, and particularly nasopharyngeal carcinoma, occur in distinct populations with endemic patterns of incidence. As a possible contributing factor is the prevalence of specific variants of EBV in distinct geographical locations with unique biological or pathological properties, strain variation has been studied continuously (Bornkamm *et al.*, 1980; Raab-Traub *et al.*, 1980). Most biological studies of EBV have involved virus obtained from Burkitt's lymphomas (HR1, W91, Akata, AG876) or from a patient with infectious mononucleosis (B95-8); one viral strain was obtained from a nasopharyngeal carcinoma biopsy sample that was co-cultured with lymphocytes (Crawford *et al.*, 1979). Viral replication could be induced in some of the lymphoid cell lines, and these studies revealed that virus from nasopharyngeal carcinomas is both replication and transformation competent. This isolate, the MABA strain, was further compared by restriction enzyme analysis and shown to be largely identical to other EBV strains from cases of infectious mononucleosis and Burkitt's lymphoma (Bornkamm *et al.*, 1980; Polack *et al.*, 1984b). EBV DNA in nasopharyngeal carcinoma biopsy samples was also shown to be similar to that in laboratory strains (Raab-Traub *et al.*, 1987). A second nasopharyngeal carcinoma strain was obtained by direct fusion of nasopharyngeal carcinoma tumour cells with a human adenoidal epithelial cell line (Takimoto *et al.*, 1985). This cell line, NPC-KT, produces virus that can transform lymphocytes and can spread during cell-to-cell fusion (Sato *et al.*, 1989; Yoshizaki *et al.*, 1994).

Specific genetic variations in the viral genome have been looked for by restriction enzyme polymorphism, and specific strains were found to be prevalent in different populations. Lung *et al.* (1990) and Lung and Chang (1992) first described a common Chinese variant marked by loss of the *Bam*HI site between the W1′ and I1′ fragments (designated *C* variant), frequently with an additional *Bam*HI site in the F fragment (*f* variant). This *Cf* variant was detected in 86% of nasopharyngeal carcinoma biopsy samples from southern China, while healthy subjects usually had the *C* variation without the *f* polymorphism. The *f* variant was more frequently detected in the nasopharyngeal carcinoma biopsy samples than in peripheral blood lymphocytes or throat washings (Lung *et al.*, 1992). The *C* variation was also prevalent in Japanese populations; however, this variant lacked the *f* polymorphism (Tamura *et al.*, 1993). A polymorphism at an *Xho*I site was also detected in most Chinese samples (Hu *et al.*, 1991a; Miller *et al.*, 1994b).

Abdel-Hamid *et al.* (1992) performed similar analyses on epithelial and lymphoid malignancies from endemic and non-endemic regions, including Chinese, Mediterranean and American populations, determining the restriction enzyme polymorphisms and EBV type. The prevalent strain in Southeast Asia was a variant of EBV-1, which had the *Bam*HI and *Xho*I polymorphisms described above. In nasopharyngeal carcinomas from areas of middle or low incidence, a distinctive EBV-1 subtype, originally identified in the nasopharyngeal carcinoma-derived MABA strain, was found in approximately 50% of the tumours, and an additional 25% had the Chinese polymorphisms. In the Alaskan Inuit population, a new variant of EBV-2, with the polymorphisms from the right end of the genome that distinguish the Chinese EBV-1 strain, was identified in nasopharyngeal carcinomas and carcinomas of the parotid gland. In contrast, the standard EBV-1 genome, lacking the above-mentioned polymorphisms, was detected in lymphomas from central Africa (Burkitt's lymphoma), Egypt and the United States.

The presence of specific restriction enzyme polymorphisms suggested that EBV strains might have consistent sequence variation. As EBV *LMP-1* is essential for transformation and is considered to be the EBV oncogene, many studies have focused on strain variation in the *LMP-1* gene. The sequence of a Chinese isolate, Cao, revealed several amino-acid changes and deletion of 10 amino acids within LMP-1 (Hu *et al.*, 1991a). The sequence of 50 Taiwanese isolates revealed that a particular strain was prevalent in Taiwan and had greater transforming ability in BALB/c 3T3 cells than LMP-1 from B95-8 (Chen *et al.*, 1992a). A similar comparison of the transforming ability of B95-8 and Cao in the RHEK epithelial cell line showed that both increased proliferation, with higher colony-forming efficiency and decreased differentiation, marked by decreased involucrin expression. The B95-8 and Cao transformants differed morphologically, and only the Cao transformants induced tumours in nude mice (Hu *et al.*, 1993; Zheng *et al.*, 1994c).

Sample *et al.* (1994) found that the *LMP-1* sequence in the prototype EBV-1 and EBV-2 strains were nearly identical. A comprehensive analysis of *LMP-1* sequence variation in nasopharyngeal carcinoma isolates from several geographical regions revealed that consistent base-pair changes, including the 10-amino acid deletion, marked a Chinese EBV-1 strain. In contrast, the Alaskan strain had many of the same base-pair changes but was undeleted and represented EBV-2. This study also showed that the number of small-repeat elements within *LMP-1* can vary, such that viral replication could produce progeny with differing numbers of the small *LMP-1* repeat (Miller *et al.*, 1994a). In a comparison of the EBV strain in nasopharyngeal carcinoma, T-cell lymphoma, normal nasopharynx and throat washings in Taiwan, the Chinese prototype strain was detected in 48/48 nasopharyngeal carcinoma samples, 23/25 samples of normal nasopharynx and 37/44 throat washings, indicating the widespread prevalence of this strain in Taiwan (Chang *et al.*, 1995).

Many studies have focused on the small deletion in *LMP-1*. The effect of the deletion on BALB/c 3T3 cell transformation was analysed by producing chimaeric proteins (Li *et al.*, 1996). It was found that the enhanced transforming ability of the Chinese strain in BALB/c 3T3 cells was transferred with the carboxy terminus that included the 10-amino acid deletion. Deletion of the 10 amino acids in B95-8 LMP-1 resulted in the ability to

induce transformation in BALB/c 3T3 cells and tumorigenicity, while insertion of the 10 amino acids into the Chinese strain eliminated transformation and tumorigenicity. As B95-8 *LMP-1* itself transforms in rodent fibroblasts, the significance of these differences in the transforming ability of *LMP-1* in cell lines remains unclear.

The presence or absence of the deleted form of *LMP-1* and multiple sequence changes in various disease states have also been evaluated (Knecht *et al.*, 1996). Some studies suggest that strains with deleted *LMP-1* are found in more aggressive tumours, and others indicate that these changes reflect the prevalence of a given strain within a population (Knecht *et al.*, 1993; Cheung *et al.*, 1996: Khanim *et al.*, 1996). In epithelial tumours, specific amino-acid changes have been detected in EBNA-1 which are suggested to influence its biological properties (Snudden *et al.*, 1995).

Trivedi *et al.* (1994) studied the effect of *LMP-1* on the immunogenicity of mouse mammary carcinoma cells. Immunization with B95-8 *LMP-1*-transfected tumour cells protected against challenge with B95-8 LMP-1-positive tumour cells, whereas immunization with Cao LMP-1 did not. This difference could be due to an inability of this particular mouse strain to present the MHC class-I or class-II epitopes of the Cao strain. It is possible that similar differences in immune response occur in distinct human populations.

These studies suggest that different EBV strains predominate in different geographical areas and that some variants may be detected preferentially in nasopharyngeal carcinoma. The prevalence of these distinct strains in epithelial malignancies could reflect an epithelial cell tropism in which particular variants of EBV preferentially establish latent infection in epithelial cells, or it may reflect differences in immunogenicity. It is also possible that strains that encode *LMP-1* with increased transforming properties have increased pathogenicity *in vivo.*

4.5.2 *Contribution of environmental and genetic factors*

4.5.2.1 *Dietary cofactors*

(*a*) *Experiments in rodents*

Groups of 10 male and 10 female Wistar WA rats were fed salted fish for six months and observed for one to two years. Four female rats developed carcinomas in the nasal or paranasal regions, while none was observed in the six control rats (Huang *et al.*, 1978c).

Salted fish purchased weekly in streetside markets in Hong Kong was fed to 111 male and 110 female Wistar-Kyoto rats aged 21 days, immediately after weaning in order to recreate the human experience. The animals were randomized by sex into one of three experimental groups: groups of 37 males and 37 females were fed a powdered diet consisting of one part steamed salted fish and three or five parts certified rat chow for the first 18 months; 37 males and 36 females were given rat chow only throughout the experiment. After 18 months, all rats were given rat chow pellets for the remainder of their lifespan. Four malignant nasal cavity tumours developed among the treated rats: the first three tumours (one undifferentiated carcinoma, one moderately differentiated squamous-cell carcinoma and one spindle-cell carcinoma) all occurred in the group at the

high dose; the fourth tumour was a spindle-cell tumour (not otherwise specified) in a rat at the low dose. No tumours of the upper respiratory tract were observed among control rats, consistent with the rarity of reports of such spontaneous tumours in rats. The rate of tumour occurrence among treated rats was statistically different from the base rate of zero ($p = 0.02$). The levels of salted fish fed to the rats were close to the range of human consumption, i.e. the amounts fed to Cantonese babies during weaning, as determined in a survey of mothers in Guangzhou, China (Yu *et al.*, 1989b).

In a two-year experiment, 16 pregnant Sprague-Dawley rats were randomized into four groups. Four rats were fed a diet consisting of 10% by weight steamed salted fish (purchased in Guangzhou, China) throughout pregnancy and lactation, and the 41 offspring continued to be fed the diet after weaning. Four pregnant rats were fed standard rat chow during pregnancy and lactation, but their 41 offspring were fed the 10% fish diet after weaning. Rats in the third group received a diet consisting of 5% salted fish by weight, as for the first group. Rats in the last group served as controls, both the mothers and the 40 offspring being fed a standard rat chow diet throughout the experiment. Seven nasal tumours were observed among the rats fed salted fish, with four in group 1 (one squamous-cell carcinoma, one poorly differentiated carcinoma, one fibrosarcoma and one adenocarcinoma), two in group 2 (one squamous-cell carcinoma and one rhabdomyosarcoma) and one soft-tissue sarcoma in group 3; no respiratory-tract tumours were seen in the controls. The difference in the occurrence of nasal tumours in the four experimental groups was statistically significant (p for linear trend = 0.04) (Zheng *et al.*, 1994d).

(*b*) *High-risk populations*

Low levels (subparts per million) of several volatile nitrosamines, including *N*-nitrosodimethylamine, *N*-nitrosodiethylamine, *N*-nitrosodi-*n*-propylamine, *N*-nitrosodi-*n*-butylamine and *N*-nitrosomorpholine, have been detected in samples of Chinese salted fish (Huang *et al.*, 1981; Tannenbaum *et al.*, 1985). Most of these volatile nitrosamines can induce nasal tumours in animals (Haas *et al.*, 1973; Pour *et al.*, 1973; Althoff *et al.*, 1974; Lijinsky & Taylor, 1978). Bacterial mutagens were detected in Chinese salted fish that had been exposed to a nitrosating agent under simulated gastric conditions (Tannenbaum *et al.*, 1985; Weng *et al.*, 1992). Directly acting genotoxic substances have also been found in extracts of this food (Poirier *et al.*, 1989). In addition, samples of Chinese salted fish were found to contain substance(s) capable of activating EBV in latently infected Raji cells (Shao *et al.*, 1988; Poirier *et al.*, 1989).

Low levels (parts per billion) of several volatile nitrosamines have been detected in samples of Chinese mustard green, *chung choi* and fermented soya-bean paste and in Tunisian *quaddid* and *touklia* (Poirier *et al.*, 1987). *N*-Nitrosodimethylamine was detected in *quaddid*, *touklia*, salted mustard greens and *chung choi*; *N*-nitrosopiperidine in *touklia* and salted mustard greens; and *N*-nitrosopyrrolidine in *quaddid*, *touklia*, salted mustard greens, *chung choi* and fermented soya-bean paste. Directly acting genotoxic substances have been found in samples of Chinese salted shrimp and fermented soya-bean paste and in Tunisian *quaddid*, *touklia* and *harissa* (Poirier *et al.*, 1989). In addition, samples of *harissa* and *quaddid* were shown to contain substances capable of activating

EBV in latently infected Raji cells (Shao *et al.*, 1988; Poirier *et al.*, 1989). These substances have been characterized as including macromolecular lignin which can activate the *ZEBRA* EBV latent gene (Bouvier *et al.*, 1995).

Zheng *et al.* (1993) compared the levels of urinary nitrate and four nitrosamino acids in 77 healthy subjects in two villages in southern China in which there is a 10-fold range in the incidence of nasopharyngeal carcinoma. The five urinary metabolites were measured in 12-h samples of urine collected in the absence of treatment, after ingestion of L-proline in the urine of subjects from the high-risk village or after ingestion of L-proline in combination with vitamin C. The total urinary nitrosamino acids in the urine of subjects from the high-risk village was significantly increased above the baseline level after proline treatment, while no difference from background levels was noted after treatment with both proline and vitamin C. No such variation in nitrosamino acid levels was found in subjects in the low-risk village. The mean urinary nitrate levels were significantly higher among subjects in the high-risk than the low-risk village in both treated and untreated urine samples. These results demonstrate a higher potential for endogenous nitrosation in subjects living in areas of high risk for nasopharyngeal carcinoma and suggest the presence of nitrosation inhibitors in the diet of southern Chinese living in areas at relatively low risk for nasopharyngeal carcinoma. These results also support the hypothesis that dietary precursors of nitroso compounds are involved in the pathogenesis of nasopharyngeal carcinoma.

4.5.2.2 *Genetic factors*

The role of HLA-related genes in nasopharyngeal carcinoma is discussed in section 2.4.3. Several HLA types have been associated with development of the disease, and familial aggregation has been linked to the HLA region (Chan *et al.*, 1983b; Lu *et al.*, 1990). A recent study revealed that the CTL presentation of LMP-2 was restricted to HLA A2.1 (Murray *et al.*, 1992b), and an inverse association between risk for nasopharyngeal carcinoma and the HLA A2 type has been described (Burt *et al.*, 1994). It has also been suggested that patients with nasopharyngeal carcinoma have impaired T-cell immunity to EBV in general (Tamura *et al.*, 1992); however, if impaired T-cell immunity and HLA-restricted presentation contribute to the development of nasopharyngeal carcinoma, an elevated incidence of this tumour would be expected in patients with HIV, which has not been observed to date (Melbye *et al.*, 1996).

High-risk families in which multiple cases of nasopharyngeal carcinoma have occurred have been described in both high- and low-risk populations (for details, see section 2.4.3.3). Familial clustering of nasopharyngeal carcinoma is likely to be the result of genetic constitution and environmental exposures.

Genetic changes characteristic of other malignancies, such as *c-myc* rearrangement, *p53* mutation, *Rb* alterations or *ras* mutations, would not necessarily contribute to altered growth, and such mutations have not been detected in nasopharyngeal carcinomas in Chinese, American or Arab populations (Effert *et al.*, 1992; Spruck *et al.*, 1992; Sun *et al.*, 1993; Nasrin *et al.*, 1994; Sun *et al.*, 1995). Unmutated p53 protein is, however, detected at high levels in nasopharyngeal carcinomas and *p53* expression has been shown

to be induced by NFκB, a transcription factor that is activated by *LMP-1* (Niedobitek *et al.*, 1993b; Chen & Cooper, 1996). In studies to investigate whether an EBV protein interferes with some aspect of *p53* function and eliminates a selection for inactivating mutations, it was found that the EBV BZLF1 protein binds p53 and inhibits p53-dependent transcriptional *trans*-activation, however, *BZLF1* is expressed in rare cells in nasopharyngeal carcinomas and would be unlikely to influence the selection of inactivating mutations in *p53* in the majority of nasopharyngeal carcinoma cells (Zhang *et al.*, 1994b). In latent infections, EBV does not interfere with the ability of *p53* to arrest cells in G1 after DNA damage by inducing expression of the *p21* cyclin kinase inhibitor. Two studies have shown, however, that LMP-1 inhibits p53-mediated apoptosis induced by serum withdrawal (Okan *et al.*, 1995; Fries *et al.*, 1996). This property could be due to induction of *bcl2*, which has been described in lymphoid cell lines (Henderson *et al.*, 1991); but elevated *bcl2* expression is not linked to EBV infection in epithelial tumours (Lu *et al.*, 1993) and *bcl2* was not induced in epithelial cell lines expressing LMP-1 that were protected from apoptosis (Fries *et al.*, 1996). Specific protection from p53-mediated apoptosis was conferred by the A20 protein, which is induced by LMP-1 expression and protects against apoptosis induced by tumour necrosis factor or serum withdrawal. Protection against p53-mediated apoptosis is likely to be responsible for the lack of *p53* mutations in EBV-associated cancers.

Recent studies have identified areas of loss of heterozygosity on several chromosomes, including the regions 3p24 and 9p21 (Huang *et al.*, 1991, 1994). Deletions at 3p24 have also been detected in nasopharyngeal carcinomas in Indian populations (Kumari *et al.*, 1995). The *p16* gene at 9p21 is completely deleted in some samples of nasopharyngeal carcinoma (Lo *et al.*, 1995). Although *p16* is not deleted in the C15 tumour passaged in nude mice, it is not expressed (Sun *et al.*, 1995). The *p16* gene is a critical regulator of cell-cycle progression through G1, and mutations in the *Rb* gene, amplification of cyclins or inactivation of cyclin-dependent kinases can all inactivate this component of cellular control. Deletion of *p16* and repression of *p16* expression in nasopharyngeal carcinomas suggests that this pathway is affected in this tumour, as in many others. The identification of other specific genes in the regions that show chromosomal loss may help in finding the critical cellular genes that contribute to the development of nasopharyngeal carcinoma in high-risk populations or a gene that is affected by mutagenic environmental factors.

4.6 Other malignancies, including lymphoepithelial carcinomas

Latent EBV infection has been detected in gastric lymphoepithelial carcinoma, and EBV is also found in a subset of gastric adenocarcinomas in all geographic areas in which it has been studied (Shibata & Weiss, 1992; Rowlands *et al.*, 1993; Selves *et al.*, 1996a). Clonal EBV is detected in the tumours, while immunohistochemical studies suggest that EBNA-1 is expressed in the absence of detectable EBNA-2 or LMP-1 in both adenocarcinomas and lymphoepithelial carcinomas (Rowlands *et al.*, 1993; Fukayama *et al.*, 1994; Imai *et al.*, 1994a; Ott *et al.*, 1994; Murray *et al.*, 1996; Selves *et al.*, 1996b). This suggests a type-I EBV latency rather than the type-II latency found in

nasopharyngeal carcinomas. These results were confirmed recently at the transcriptional level by RT-PCR, which showed that EBNA-1 is expressed from the Q promoter, as in types-I and -II latency (Sugiura *et al.*, 1996). Consistent with the results of previous immunohistochemical studies, *EBNA-2* and *LMP-1* mRNA were not detectable; however, transcripts derived from the *Bam*HI A fragment of the viral genome were detected in all cases, and a proportion of cases had detectable levels of *LMP-2A* mRNA (Sugiura *et al.*, 1996). In a preliminary immunohistochemical study with new LMP-2A-specific monoclonal antibodies, no staining was detected in EBV-positive gastric carcinomas (Niedobitek *et al.*, 1997c). This suggests that carcinomas may represent a form of type-I latency, with possible additional expression of LMP-2A in a proportion of cases.

Expression of the BZLF1 protein and of *BZLF1* transcripts has also been reported in a few scattered cells in a proportion of gastric carcinomas (Niedobitek *et al.*, 1992b; Rowlands *et al.*, 1993), while Sugiura *et al.* (1996) did not detect lytic cycle RNA transcripts by RT-PCR. This suggests that viral replicative genes are expressed in a very small fraction of tumour cells; however, it is presently unclear whether this leads to the production of infectious virions. In a single case, Niedobitek *et al.* (1992b) reported the absence of detectable gp350 expression in a lymphoepithelial carcinoma of the stomach.

Multiple other types of epithelial cancer have also been linked to EBV. Most are rare undifferentiated carcinomas with a prominent lymphoid stroma (lymphoepithelial carcinomas), reminiscent of nasopharyngeal carcinoma. EBV has been associated with lymphoepithelial carcinoma of the parotid gland (Saemundsen *et al.*, 1982; Saw *et al.*, 1986; Huang *et al.*, 1988; Raab-Traub *et al.*, 199l), which develops at relatively high incidence among Inuit populations and in Chinese (Saw *et al.*, 1986; Huang *et al.*, 1988). Other epithelial salivary-gland tumours have been shown to be EBV-negative (Wen *et al.*, 1997). In a Japanese study, EBV was detected in lymphoepithelial carcinoma samples but not in benign lymphoepithelial lesions (Nagao *et al.*, 1996), suggesting that EBV infection is the pivotal step to malignancy. Like nasopharyngeal carcinoma, undifferentiated parotid tumours contain clonal EBV and express EBERs, LMP-1 and *Bam*HI A transcripts (Raab-Traub *et al.*, 1991).

Other rare cancers are linked to EBV infection. These include a subset of undifferentiated sinonasal carcinomas in Italian and Chinese populations (Gallo *et al.*, 1995; Leung *et al.*, 1995b), lymphoepithelial cholangiocarcinoma (Hsu *et al.*, 1996) and smooth-muscle tumours in HIV-infected children and transplant recipients (Morel *et al.*, 1996). The smooth-muscle tumours express *EBER-1* RNA and *EBNA-2* but not *LMP-1* (Lee *et al.*, 1995a). In all samples, identification of the EBV termini showed that the infection was non-permissive and clonal with regard to EBV. Genes expressed in latency type II are found in most tumours, although the detection is variable and some tumours, such as gastric carcinoma, may express only *EBNA-1*. The detection of EBV in tumours of diverse cell types indicates that EBV may gain entry into different cell types under specific circumstances and can potently affect cell growth. The clonality of EBV suggests that establishment of a latent transforming infection is the rare event that triggers cancer development.

4.7 Immune responses and EBV-associated malignancies

A common denominator of virus-associated tumours is the persistence in the malignant cells of all or parts of the viral genetic material and the continued expression of viral proteins that are the potential targets of tumour-specific rejection. The various patterns of viral gene expression identified in EBV-associated malignancies (see Table 3) and the diverse cellular origin of the malignant cells themselves could provide different types of challenge to the host immune system. Thus, failures or specific changes in the host immune response are likely to play different roles in the pathogenesis of these tumours. Characteristic changes in the pattern of antibodies to the latent antigens and to antigens associated with the productive cycle occur in patients with EBV-associated tumours. These antibody patterns reflect the load of infectious virus and/or viral antigen-expressing cells, and their role in limiting the growth of virus-infected cells is uncertain. With the exception of immunosuppressed patients who develop EBV-associated lymphomas, little is known about the status of cell-mediated virus-specific immune responses in patients with EBV-carrying tumours.

4.7.1 *B-Cell lymphoma and other tumours associated with severe immunosuppression*

The nine viral antigens that are typically detected in lymphoblastoid cell lines are also expressed in B-cell lymphomas arising in immunosuppressed patients (see Table 3). The phenotypic similarity of these lymphomas to lymphoblastoid cell lines suggests that the lymphomas represent virus-derived lesions growing out opportunistically in the absence of immune control. Indeed, regression has been reported after cessation of immunosuppressive therapy in renal transplant recipients (Starzl *et al.*, 1984), and passive transfer of blood lymphocytes or activated EBV-specific CTL cultures *in vitro* has been successfully used in the treatment of lymphomas arising in bone-marrow recipients (see section 1.5.3; Papadopoulos *et al.*, 1994; Rooney *et al.*, 1995). Immunosuppression could also play a role in the pathogenesis of EBV-positive leiomyosarcomas, since these tumours appear to express at least one highly immunogenic viral antigen (see Table 3).

4.7.2 *Burkitt's lymphoma*

Although transient impairment of EBV-specific responses has been demonstrated during acute malaria (Whittle *et al.*, 1984), normal levels of EBV-specific CTL precursors were demonstrated in a single study of the inhibition of autologous B-cell transformation in Burkitt's lymphoma patients (Rooney *et al.*, 1997). Even so, disturbance of the antiviral response is likely to play an important role in the pathogenesis of endemic Burkitt's lymphoma. EBV infection early in life and recurrent episodes of severe malaria were shown to increase the risk for the lymphoma in African children (reviewed by Magrath, 1990). Malarial infection is associated with chronic B-cell stimulation and follicular hyperplasia, which is likely to favour the establishment of a large pool of latently infected B lymphocytes from which cytogenetically altered cells may arise during the rapid proliferative phase of follicular reactions. A similar mechanism could also explain the high incidence of Burkitt's lymphoma in HIV-positive patients, in whom chronic stimulation of the B-cell compartment is a common feature in the pre-AIDS

stage. EBV-positive Burkitt's lymphoma lines that have maintained *in vitro* the phenotypic characteristics of the tumour *in vivo* are insensitive to lysis by EBV-specific MHC class I-restricted CTLs (Rooney *et al.*, 1995). This observation is consistent with the finding that the viral antigens that are recognized by these effectors are down-regulated and only EBNA-1 is expressed in the tumour. EBNA-1 may be protected from MHC class I-restricted rejection responses by an inhibitory effect of the glycine–alanine repeat on antigen processing (Levitskaya *et al.*, 1995). MHC class II-restricted EBNA-1-specific CTLs have been demonstrated *in vitro* (Khanna *et al.*, 1996), but the capacity of these cells to recognize the tumour is not firmly established. In addition to down-regulation of viral antigens, Burkitt's lymphoma cells consistently show low expression of adhesion molecules and MHC class I-restricted antigens and a selective loss of certain class-I alleles, with HLA A11 as the most consistent example (Masucci *et al.*, 1987; Andersson *et al.*, 1991). These features are characteristic of both EBV-positive and EBV-negative tumours and may therefore represent a phenotypic feature of the Burkitt's lymphoma precursor (Torsteinsdottir *et al.*, 1989). At least some may be specifically induced by the constitutive activation and overexpression of *c-myc* that characterizes this tumour (Polack *et al.*, 1996). More recently, down-regulation of the transporters associated with antigen presentation (Khanna *et al.*, 1995; Rowe *et al.*, 1995) and defects of antigen processing (Frisan *et al.*, 1996) were reported to be consistently associated with the Burkitt's lymphoma-cell phenotype.

4.7.3 *Hodgkin's disease*

The role of specific immunosuppression in the pathogenesis of Hodgkin's disease has been discussed extensively(Slivnick *et al.*, 1990). A decrease in EBV-specific responses, as assessed by the regression assay, was suggested in earlier studies; however, recent reports clearly demonstrate the presence of EBV-specific CTL precursors in the blood of Hodgkin's disease patients, independently of the EBV status of the tumour (Dolcetti *et al.*, 1995; Frisan *et al.*, 1995). A comparative study of EBV-specific responses in patients with EBV-positive and EBV-negative Hodgkin's disease failed to demonstrate virus-specific cytotoxicity within the tumour-infiltrating lymphocytes of six EBV-positive cases, while EBV-specific effectors were readily detected (Frisan *et al.*, 1995). The detection of CTLs in the blood of patients with EBV-positive Hodgkin's disease suggests that virus-specific reactivity is selectively suppressed in the tumour. The finding of multiple sequence variations in the *LMP-1* gene in EBV isolates from cases of Hodgkin's disease and nasopharyngeal carcinoma supports the possibility that the tumour cells present altered peptides that act as antagonists of EBV-specific responses. Alternatively, production of IL-10 in the LMP-1-positive tumours (Herbst *et al.*, 1996b) may mediate local inhibition of CTL responses.

4.7.4 *Nasopharyngeal carcinoma*

Nasopharyngeal carcinoma arises in apparently immunocompetent individuals, but the frequency is not increased in patients with recognized forms of immunosuppression. Lymphocytes from nasopharyngeal carcinoma patients maintain the capacity to inhibit

the proliferation of autologous virus-infected cells in regression assays, although a significant impairment in virus-specific T-cell immunity is shown in newly diagnosed patients when compared with either long-term survivors or controls (Moss *et al.*, 1983b). It is therefore quite surprising that the continuous expression of at least two highly immunogenic viral antigens, LMP-1 and LMP-2, does not lead to rejection. A characteristic feature of tumours expressing LMP-1 is the presence of abundant lymphoid infiltrate, which may be indicative of an on-going reaction to viral and/or tumour antigens. Indeed, LMP-1 was shown to increase the stimulatory capacity of EBV-negative Burkitt's lymphoma lines in allogeneic mixed-lymphocyte cultures (Cuomo *et al.*, 1990), to up-regulate the expression of adhesion molecules (Wang *et al.*, 1990) and to enhance the presentation of endogenous and exogenous antigens (de Campos-Lima *et al.*, 1993b; Rowe *et al.*, 1995). It is not known whether LMP-1 has similar effects on the immunogenic phenotype of epithelial cells, and the sensitivity of these cells to EBV-specific CTLs has not been investigated. Several surface molecules expressed in undifferentiated nasopharyngeal carcinoma cells, such as CD70, CD80 and CD86, may be involved in the induction of T-cell activation, and T cells with an activated phenotype have been detected in the tumour; however, their role in the pathogenesis of the disease remains unclear (Agathenanggelou *et al.*, 1995). A study of the expression of the T-cell receptor repertoire of tumour-infiltrating lymphocytes from nasopharyngeal carcinomas showed a significantly lower representation of certain V families (Vα10, Vα11, Vα13, Vα14, Vβ14 and Vβ20) than in control biopsies. Some of these differences were also observed in peripheral blood (Chen *et al.*, 1995b). Of particular interest is the observation that the frequencies of Vα17 ($p = 0.01$) and Vα18 were significantly lower ($p = 0.04$) in HLA B46-positive nasopharyngeal carcinoma patients than in B46-positive controls. It is presently unknown whether specific EBV peptides are present through HLA B46. Taken together, these results suggest that the expression of certain HLA alleles (see section 1.1.5) and the absence of certain T-cell receptor Vα/β families is important in the pathogenesis of nasopharyngeal carcinoma.

An additional clue is provided by comparisons of the immunogenicity of a nasopharyngeal carcinoma-derived LMP-1 with LMP-1 from the standard EBV laboratory strain B95.8 in a transfectant mammary carcinoma model in mice. While transfectants expressing the B95.8-derived LMP-1 became immunogenic and were rejected by syngeneic animals, the nasopharyngeal carcinoma-derived polypeptide failed to induce rejection (Trivedi *et al.*, 1994). Conceivably, the tumour-derived protein lacks antigenic epitopes that are relevant for rejection or fails to induce a phenotypic shift that would increase immunogenicity.