Non-proliferative Interactions of Epstein-Barr Virus and Human B Lymphocytes

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Epstein-Barr virus (EBV) and man have a long common history, during which both evolved strategies leading to a largely harmless coexistence (Klein, 1994; Rickinson and Kieff, 2001). The virus was detected in 1965, in cultured cells of an African Burkitt lymphoma (BL). Its capacity to transform and immortalize human B lymphocytes *in vitro* was shown soon thereafter and therefore it was assumed that the lymphoma is induced by EBV (Küppers, 2003). However, the discovery of EBV-negative lymphomas with similar pathology and carrying a for BL common, typical cytogenetic marker indicated that even if the virus can contribute to the development of this malignancy, it is not responsible for proliferation of the tumour cells. The cytogenetical change is a reciprocal chromosomal translocation between chromosome 8 and either chromosome 14, 2 or 22. In such cells the expression of the *myc* oncogene (chr. 8) is constitutive due to its regulation by the juxtaposed immunoglobulin (heavy, chr 14, or light chains, chr 2 or 22) genes. The active *myc* gene drives the cells for division. Whether and how EBV can have a role in the aetiology of BL is still not clarified.

EBV infection

Serological studies revealed that EBV infection is almost ubiquitous in humans. In developing countries and in low socioeconomic groups, infection is acquired in childhood. In children, disease is caused rarely, while half of the infected adolescents or adults develop infectious mononucleosis (IM), a benign self-limited lymphoproliferation. The clinical picture is highly variable, but even if it is severe, it always subsides. Infection – irrespective whether or not it is followed by the specific symptoms – is always followed by a lifelong viral carrier state. This is easily detectable by the sustained virus-specific immunity, humoral and cell-mediated, directed against viral structural proteins and against virally encoded proteins that are expressed in the infected cells. A prolonged disease state, designated as chronic active EBV infection, occurs rarely. In a very rare hereditary condition, X-linked lymphoproliferative (XLP) disease, EBV infection can either be fatal (half of the cases) or it leads to lymphoma development (Gilmour and Gaspar, 2003). The genetical change is mutation in a gene coding for an adaptor molecule SAP, which is part of the signalling system of lymphocyte activation. The manifestation of the disease includes a defect in T- and natural killer (NK)-cell responses. The function of the SAP protein, its role in the pathogenesis of XLP is still not clarified.

We thus carry a virus that can induce proliferation of B lymphocytes *in vitro*. For understanding the largely harmless EBV-human relationship two questions are relevant:

1. How is proliferation of EBV-infected B cells avoided *in vivo*?
2. How does the virus achieve persistence?

These questions can be answered on the basis of knowledge about the different types of EBV-B-lymphocyte interactions. *In vitro* results and certain disease conditions have ascertained that undisturbed immune functions are critical for the healthy virus carrier state (Klein, 1994; Rickinson and Moss, 1997). While many details are already clarified, new facts still emerge about the host-virus, and cell-virus interactions.

The expression of viral genes in the B lymphocyte varies. From being a harmless passenger, it can interfere with the maturation of the cell and it can also induce proliferation. Depending on the expression of the viral proteins, type I, type II and type III patterns have been distinguished.

Answer to question 1: In the virus carrier individuals, the potentially serious danger, i.e. malignant proliferation of virus-carrying B cells, is inhibited by specific immunity directed aginst the virally encoded transforming proteins.

Answer to question 2: The virus genome is maintained in resting B cells, in which it does not express the growth transformation-associated immunogenic proteins.

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Abbreviation: BL – Burkitt lymphoma, CLL – chronic lymphocytic leukaemia, EBV – Epstein-Barr virus, HD – Hodgkin's disease, HL – Hodgkin's lymphoma, H/RS – Hodgkin and Reed/Sternberg, IM – infectious mononucleosis, LMP-1 – latent membrane protein 1, NK – natural killer, PTLD – post-transplantation lymphoproliferative disease.

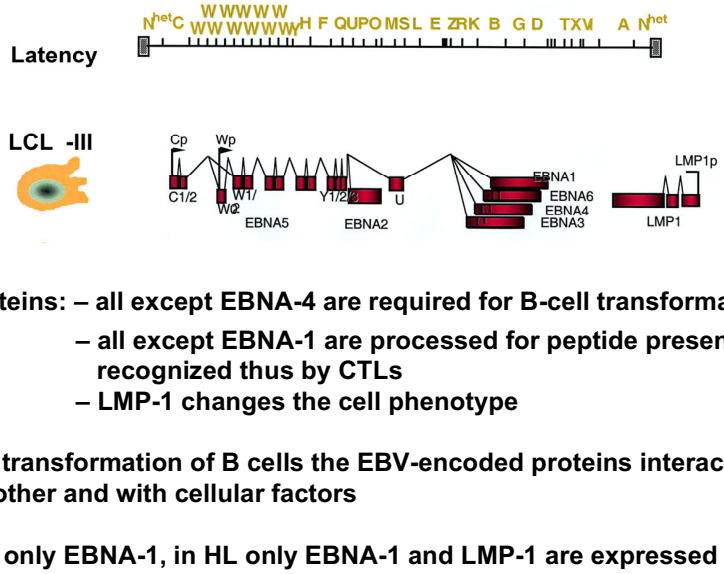

Fig. 1. EBNA and LMP-1 transcripts in EBV-transformed LCL

Expression of EBV-encoded genes

The EBV-encoded genes expressed in B cells with latent infection were defined in the *in vitro* transformed B-lymphocyte lines, lymphoblastoid cell lines (LCL). In these, the EBV genome resides as covalently closed circles, episomes. The cells express nine virally encoded proteins, six nuclear (the EBNAs) and three integral membrane proteins (the LMPs). This expression pattern is called type III or growth programme. Since only very rare cells enter the lytic cycle, the genes that are expressed in LCL are often designated "latent genes" (Young and Murray, 2003). Five of the virally encoded proteins detected in LCLs are involved in induction and in the maintenance of the transformed phenotype. They interact with each other and with cellular transcription factors. The critical genes for the transformed phenotype are *EBNA-2* and *LMP-1* (latent membrane protein-1) (Kaye et al., 1993). In B lymphocytes the expression of LMP-1 is regulated by the EBV nuclear proteins, most importantly EBNA-2, which in cooperation with cellular transcription factors, among them PBP-Jk, PU.1, CBP/p300, creates the LMP-1 promoter activating complex (Johannsen et al., 1995). Importantly, cells with type III expression pattern can be found in the malignant post-transplantation lymphoproliferative disease (PTLD) (Bräuninger et al., 2003).

Type I expression

BL cells express only the EBNA-1 protein; this is designated as type I programme (Chen et al., 1995). This viral programme does not induce cell proliferation *in vivo* or *in vitro*. As mentioned above, tumorigenesis is due to the chromosomal rearrangement and these tumour cells can grow *in vitro*. These patients are fully immunocompetent and the BL cells exist in the presence of EBV-specific immunity. This is ensured by two characteristics of the

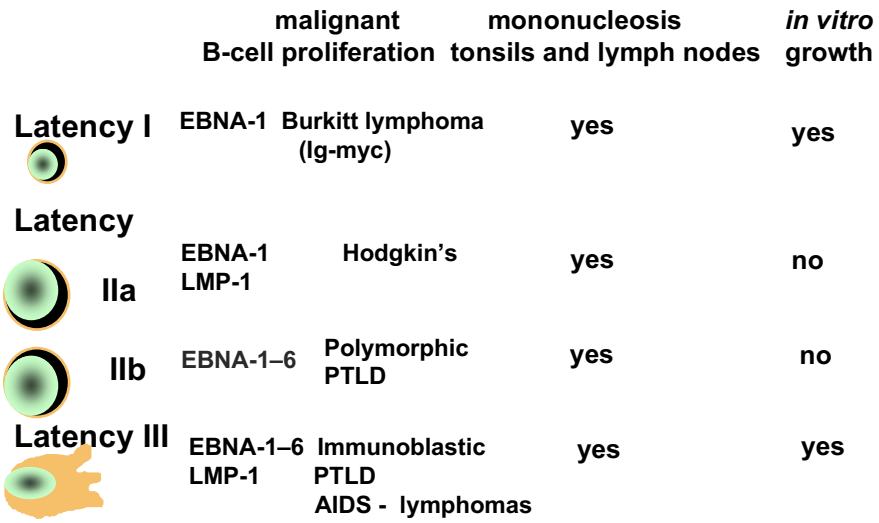

Fig. 2. Variation in the expression of EBV latent genes

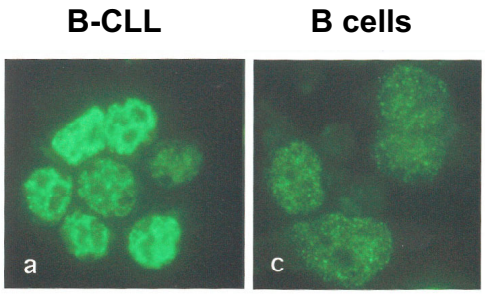

Fig. 3. EBNA-2 staining of B-CLL and blood-derived B cells. Note the appearance of CLL cells, the nuclei are smaller and the EBNA-2 distribution is coarse. The CLL cells are not activated by the EBV infection.

cells. First, the EBNA-1 protein does not provide HLA class I-associated peptides, the antigen recognition complex for the specific T lymphocytes. Secondly, the BL cell phenotype corresponds to resting, non-activated lymphocytes. Such cells do not express co-stimulatory molecules, which are needed for interaction with the T lymphocytes.

Type IIa and type IIb expression

Two additional viral expression patterns exist in cells of the B lineage. In these the EBV-encoded proteins occur in different combinations: EBNA-1 and LMP-1 expression was first discovered in nasopharyngeal carcinoma, NPC, and designated as type II programme. In order to accommodate another variation of the expressed proteins, we change its name now for type IIa. It also occurs in Hodgkin's lymphomas (HL), and in T and NK lymphomas. EBNA-2 has been shown to regulate the activation of the LMP-1 promoter in B cells, therefore the type IIa pattern was unexpected and its molecular mechanism is not yet known.

The type IIb pattern was first seen when B-chronic-lymphocytic leukaemia, B-CLL cells were infected *in vitro* with EBV. The cells express all the tested nuclear proteins, EBNA-1, 2, 4, 5, 6, but not LMP-1. Only very rare CLL clones transform to immortalized lines (Takada et al., 1980). However, when they do, their phenotype corresponds to the LCLs generated from normal B lymphocytes and then their EBV protein expression pattern is type III.

Importantly, B lymphocytes with these four EBV protein expression types were detected in lymph nodes and tonsils of IM patients and in tissues of the EBV-positive lymphoproliferations that develop in immunosuppressed patients (Niedobitek et al., 1997, Spieker et al., 2000; Kurth et al., 2000; Bräuninger et al., 2003). Populations of type IIa and type IIb cells cannot be isolated from the tissues. For studies of the type IIa pattern, HL-derived cell lines and for the type IIb pattern, B-CLL cells can be used. We have found that the *in vitro* infected CLL cells differ considerably from the infected normal B-cell populations (Avila-Carino et al., 1994; Avila-Carino et al., 1997; Tomita et al., 1998; Teramoto et al., 2000; Maeda et al., 2001; Bandobashi et al. (to be published)).

Lymphoid tissues

Burkitt's lymphoma, endemic	98%
Burkitt's lymphoma, sporadic	25%
AIDS-immunoblastic lymphoma	**60%**
- in CNS	**100%**
Post-transplant lymphoma	**100%**
Hodgkin's lymphoma	50%
T-cell lymphomas	10–30%
- lethal midline granuloma	>90%

Fig. 4. EBV-associated tumours in man

Hodgkin's lymphoma

The role of EBV in Hodgkin's disease (HD) is unknown. In the tissue, the typical Hodgkin and

Lymphoid tissues	Role of EBV
Burkitt's lymphoma, endemic Burkitt's lymphoma, sporadic	? (Ig-myc translocation)
AIDS-immunoblastic lymphoma - in CNS Post-transplant lymphoma	**proliferation**, defective immune control
Hodgkin's lymphoma	**?** rescue from apoptosis ?, interaction with inflammatory cells
T-cell lymphomas - lethal midline granuloma	?

Fig. 5. EBV-associated tumours in man

Reed/Sternberg (H/RS) cells represent only a minority, about 1%, within a population of non-neoplastic, inflammatory cells. Except for rare cases, when they are T cells, the H/RS cells belong to the B lineage. Depending on geography and on the age of the patients, a fraction of the HL patients, about 50%, have EBV carrying H/RS cells. The type IIa virus-B cell interaction does not induce *in vitro* proliferation. In spite of considerable efforts, only a few lines with H/RS characteristics have been described and they are EBV-negative. Interestingly, those belonging to the T lineage are relatively more frequent. Assignment of the H/RS cells to the B-cell lineage is based on the clonal rearrangements of the immunoglobulin (Ig) heavy and light chain genes. In spite of their Ig gene rearrangement, the HD-derived B-cell lines do not express B cell-specific genes, transcription factors, and lack conventional B lymphocyte markers like CD19, CD20, Ig (Caligaris-Cappio and Hamblin, 1999; Küppers, 2002). Detailed analysis of the gene expression profile showed considerable deviations from the expected B-lymphocyte pattern, which confirms that the differentiation programme of HR/S cells is disturbed. With regard to the expression of the EBV latent genes, the lack of EBNA-2 expression is explainable by the absence or low expression of B cell-specific transcription factors, Oct-2 and BSAP in the HR/S cells. In the absence of these transcription factors, the Wp and Cp promoters that regulate EBNA 2, 4, 5, 6 expression seem to remain inactive.

The induction of LMP-1 in B cells is regulated by EBNA-2. Thus, both in NPC and H/RS cells, which lack EBNA-2, the molecular mechanisms for induction of LMP-1 differs from the B cells. EBV-positive cell lines could not be established from either of these two tumours. In our recent our recent experiments the EBV-positive subline of an EBV-negative HL cell line, established by *in vitro* infection with a virus that carries a selective trait, exhibited type I pattern, thus only EBNA-1 was expressed. Exposure of the cells to CD40L and IL-4 however induced LMP-1. This result is highly relevant for the type IIa expression of HR/S cells *in vivo*, since these stimuli can be provided by the surrounding activated T lymphocytes (Kis et al., in press).

Chronic lymphocytic leukaemia (B-CLL)

EBV is not involved in the pathogenesis of B-CLL. The disease shows the clonal expansion of B cells resulting in the population of long-lived, resting (G_0) B lymphocytes (Caligaris-Cappio and Hamblin, 1999). The cells express the EBV receptor, CD21, and can be infected with the virus *in vitro*. Experiments with several B-CLL clones (patients) showed that *in vitro* infection leads regularly to the expression of the EBNAs, but not of LMP-1. The infection does not induce the immediate-early genes, c-*myc*, *ATF-2* and c-*Jun*, the cells do not show signs of activation, they do not transform to immunoblasts, their chromatin remains dense and they do not divide (Teramoto et al., 2000; Maeda et al., 2001; Bandobashi et al., to be published). Furthermore, pRb is not phosphorylated and p27 expression does not decline. These events are induced regularly in EBV-infected normal B-cell populations. The CLL cells are not refractory for activation, as shown by their response to the CD40 ligand, which induces expression of c-Myc and entrance to the cell cycle. While CD40L overcomes the lack of the activation step after EBV infection, the cells still do not express LMP-1 and do not become immortalized (Maeda et al., 2001). Thus, the progress of EBV infection in the B-CLL cells meets two roadblocks. The first one affects the activation step. This block can be overcome by exposure to CD40L, but LMP-1 expression and immortalization is still not induced.

The fate of type IIa and type IIb cells in the IM patients is unknown. The incidence of HL is higher in individuals with a history of IM (Küppers et al., 2002). Therefore, it is possible that with contribution of host factors, the type IIa cells may create the HL tissues. Malignancies with the type IIb phenotype are not known. We have encountered an unusual patient who had an EBV-positive subclone in his CLL population. The subclone followed the dynamics of the CLL disease for several years. The recurrent populations after treatments regularly contained the EBV-positive clone (Lewin et al., 1995).

On the basis of experiments with EBV-infected CLL cells we assume that type IIb cells are not eliminated by immunological recognition, but they may succumb to apoptosis. When such cells are activated, they may aquire the type III EBV expression pattern and then they become immunogenic and will be eliminated.

Conclusions

In vitro experiments, the behaviour of tumour-derived cultured cells, together with the EBV protein expression pattern in tumours suggest that EBV induces proliferation in B cells without additional contributing factors only when they exhibit the type III protein expression. In BL cells, constitutive activation of the *myc* oncogene leads to proliferation. In HL, interaction between the HR/S and normal cells, most probably through surface contact and through cytokines, leads to the development of lymphoma.

The consequence of EBV infection in B cells is determined by their maturation state. Among the various types of viral gene expression strategies, only type III represents an immediate threat and only for the immunosuppressed individual.

For the power of immunological control of malignancy, EBV infection is a good case. Normally, the proliferation of the EBV-transformed cells is inhibited by the immune system. In patients with immunosuppressive conditions, the developing EBV-positive B-cell

proliferation can be controlled by transfer of competent, activated T lymphocytes.

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