

Enhancement of genetic instability in human B cells by Epstein–Barr virus latent infection

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The level of genetic instability, as assessed by micronucleus (MN) formation, was higher in Epstein–Barr virus (EBV)-converted B-cell lines with one copy of the EBV genome integrated in each cell than in the parental, EBV-negative, B lymphoma cells. MN induced by EBV latency, as analysed by *in situ* hybridization, contained mainly centromeric regions, indicating that the presence of EBV affects the segregation of entire chromosomes. The instability was inhibited by treatment with antioxidants. Flow cytometric analysis indicated that there was a higher basal level of peroxides in EBV⁺ cells. Direct oxidative stress caused by hydrogen peroxide (which is known to be both apoptogenic and mutagenic) enhanced the number of MN only in an EBV-converted clone. These cells were also resistant to apoptosis, as expected, suggesting that in the parental EBV cells apoptosis may efficiently eliminate cells with genetic damage. These results show for the first time a direct involvement of EBV in the induction of genetic instability, suggesting that it could contribute to tumour progression.

Introduction

Epidemiological studies have shown that Epstein–Barr virus (EBV), a member of the herpesvirus group, is associated with lymphomagenesis and nasopharyngeal carcinoma (Epstein, 1986). Although the role of EBV in cell immortalization is well established, its role in the full development of tumoral phenotypes in B cells is still unclear (Magrath, 1991).

Some authors have suggested that EBV, by allowing the clonal expansion of infected cells, as a result of its immortalization ability and its inhibition of physiological cell death (Henderson *et al.*, 1991), can indirectly facilitate tumour progression, allowing the cell to accumulate additional spontaneous genetic changes. Inhibition of apoptotic cell death is a feature of EBV-converted cell lines (Gregory *et al.*, 1991) and apoptosis is a fundamental factor in carcinogenesis (Wyllie, 1997). Impairment by mutation of some pro-apoptogenic gene products (e.g. p53) reduces the extent of apoptosis and allows the propagation of mutant cells following genotoxic damage, eventually promoting tumorigenesis (Griffiths *et al.*, 1996). Over-expression of the more general anti-apoptosis genes, *bcl-2* and *bcl-xl*, which are not specific towards DNA damage, also enhances mutagenesis via other mechanisms, for example by inhibiting the selective elimination of heavily mutated cells from the population (Cherbonnel-Lasserre *et al.*, 1996).

Other authors have suggested that EBV may directly prime genetic instability, for instance via activation of the B-cell-

specific recombinases RAG-1 and -2 (Kuhn-Hallek *et al.*, 1995), or via cycles of integration–excision of its DNA into the host genome (Wolf *et al.*, 1993; Gargano *et al.*, 1995; Gualandi *et al.*, 1995), while the expression of its latent genes could promote the excision of EBV DNA from the host genome (Wolf *et al.*, 1995).

Despite intensive studies, direct demonstration of the ‘mutagenic’ potential of EBV has not been provided.

Genetic instability is a known characteristic of transformed cells and cells carrying viral genomes, as is the case of cells infected with SV40 (Walen, 1987), transformed by adenovirus (Schamayr *et al.*, 1990) or HTLV-I (Majone *et al.*, 1993) or carrying papillomavirus DNA (Stich *et al.*, 1990). In the latter case, the increased genetic instability in papillomavirus-transformed cells was counteracted by antioxidants, suggesting that chronic oxidative stress may have a role in some cases of viral-induced genotoxicity.

The purpose of this study was to verify if the presence of EBV genome in converted human B lymphoma lines might enhance their genetic instability, as measured by the formation of spontaneous or stress-induced micronuclei (MN) in binucleated cells by the cytokinesis-block assay (Fenech and Morley, 1985; Kirsch-Volders *et al.*, 1997). The MN test allows estimation of the genotoxic effect of agents acting at each point of the cell cycle; this is an advantage in testing the action of agents whose mechanism has not been characterized.

We also investigated the possibility that endogenous, constitutive oxidative stress, elicited by the presence of EBV, may contribute to the formation of cells containing MN. Finally, we wanted to test, in our system, the role of apoptosis inhibition in causing genetic damage cells after exogenous oxidative stress. We therefore compared the genotoxic/cytotoxic effects in EBV⁻, apoptosis-prone cells, with that in EBV⁺, apoptosis-resistant cells after exposure to hydrogen peroxide.

Materials and methods

Cell lines

BL41 is an EBV⁻ B-cell line obtained from a Burkitt lymphoma (Calender *et al.*, 1987) carrying a mutant form of the p53 gene (Farrel *et al.*, 1991); it is very prone to apoptosis (unpublished). The CIA, HS1 and E2 clones were obtained after infection of the parental BL41 line with a non-defective B85-8 EBV strain. Virus filtrate from aged B95-8 marmoset cells was concentrated by centrifugation at $14\,000 \times g$ and 2×10^5 c.f.u. were added to dishes containing 10^6 cells. Cloning was achieved by limiting dilutions or by directly picking cells with a micro-needle under an inverted microscope. Clones showing the characteristic EBV⁺ phenotype (growth in clumps) were isolated. The presence of EBV and monoclonality were checked by *in situ* hybridization on metaphase spreads and by Southern analysis using the BamHI ‘W’ probe, which represents the repeat unit of the IR1 region (this is commonly involved in recombination with the human genome) (Hurley *et al.*, 1991; Gargano *et al.*, 1992).

Chemicals and media

The Ca²⁺ ionophore, ionomycin (Sigma), the topoisomerase II inhibitor, 4’-(9-acridinylamino) methanesulfon-*m*-anisidine (*m*-AMSA) (Sigma) and the alkylating agent, trimethyl tin-chloride (TMT) (Aldrich) were dissolved in

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