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Somatic mutations and activation-induced cytidine deaminase (AID) expression in established rheumatoid factor-producing lymphoblastoid cell line

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Abstract

Epstein-Barr virus (EBV) transforms human peripheral B cells into lymphoblastoid cell lines (LCLs), allowing the production of specific antibody-secreting cell lines. We and others have previously found that in contrast to peripheral blood B cells, EBV-transformed lymphoblastoid cell lines express the activation-induced cytidine deaminase (AID) gene. The opposite is true for the germinal center-specific BCL6 gene: it is expressed in adult peripheral blood B cells and is no longer expressed in LCLs. The present work extends our findings and shows that whereas AID expression is rapidly induced following EBV infection, BCL6 expression is gradually down-regulated and is fully extinguished in already established LCLs. The question of whether AID activation induces the process of somatic hypermutation (SHM) was investigated in adult-derived LCLs. It was found that the V_H gene from the rheumatoid factor-producing RF LCL (derived from a rheumatoid arthritis patient), accumulated somatic point mutations in culture. Overall, nine unique mutations have accumulated in the rearranged V_H gene since the generation of the RF cell line. Four additional intraclonal mutations were found among 10 cellular clones of the RF cells. One out of the four was in CDR1 and could be correlated with loss of antigen-binding activity in three out of the 10 clones. Altogether, these 13 mutations were preferentially targeted to the DGYW motifs and showed preference for CG nucleotides, indicating that they were AID-mediated. By contrast, mutations were not detected among 3700–4000 nucleotides each of the Vλ, Cμ and GAPDH genes derived from the same RF cell cultures and the cellular clones. Our results thus show that AID may generate point mutations in the rearranged Ig V_H during in vitro cell culture of adult-LCLs and that these mutations may be responsible, at least in part, for the known instability and occasional loss of antigen-binding activity of antibody-secreting LCLs.

Keywords: B lymphocytes; Epstein-Barr virus; Monoclonal antibodies; Rheumatoid factor; AID; Somatic hypermutation

1. Introduction

The association between Epstein-Barr virus (EBV) and a variety of B cell malignancies, nasopharyngeal carcinoma and of other tumors is well known (Niedobitek et al., 2001). In vitro, the virus transforms peripheral B lymphocytes to generate proliferating cell lines that are initially diploid, but have a limited lifespan and are non-malignant. However, at a later stage within few months following transformation, the cells can undergo an immortalization step associated with higher telomerase activity, increased telomere length, aneoploidy and tumorigenicity (Sugimoto et al., 2004).

Transformation of B cells derived from PBL by EBV can result in formation of lymphoblastoid cell lines (LCLs) and is an alternative way for the hybridoma technology to generate specific human antibody-forming cell lines (Steinitz et al., 1977). However, for unknown reasons, some of these cells may lose their ability to produce the specific antibodies. Subcloning and cell fusion procedures are being used to overcome this problem (Glasky and Reading, 1989; Kozbor and Roder, 1981; Roder et al., 1986).

We and others have recently shown that EBV infection induces the expression of the activation-induced cytidine deaminase (AID) gene in peripheral blood B cells (He et al., 2003; Levy

Abbreviations: AID, activation-induced cytidine deaminase; BL, burkitt lymphoma; CBL, cord blood lymphocytes; CSR, class switch recombination; EBV, epstein barr virus; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GC, germinal center; LCL, lymphoblastoid cell line; PBL, peripheral blood lymphocytes; RF, rheumatoid factor; SHM, somatic hypermutation

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et al., 2003). AID in mammals is specifically expressed in activated (Muramatsu et al., 1999) and germinal center (GC) B cells (Muramatsu et al., 2000) and was found to be responsible for the three known major processes that generate antibody diversity: class switch recombination (CSR), somatic hypermutation (SHM) and gene conversion which occur in the immunoglobulin (Ig) genes [rev. in Besmer et al. (2004), Harris et al. (2002), Li et al. (2004) and Neuberger et al. (2005)]. Biochemical and biological evidence strongly support the contention that the AID enzyme acts as a mutagen by converting cytidine to deoxyuracyl residues in single-strand DNA targets (Bransteitter et al., 2003; Chaudhuri et al., 2003; Dickerson et al., 2003; Ramiro et al., 2003), thereby initiating a chain of multiple base excision and mismatch repair events that lead to the generation of somatic point mutations not only in the initial dC/dG target nucleotides but also in neighboring regions, preferably at dA/dT nucleotides (Neuberger et al., 2005). The mutations are targeted to the rearranged Ig variable region genes, preferentially hitting certain hotspot motifs (Dorner et al., 1997; Rogozin and Diaz, 2004) and have an estimated rate of $\sim 10^{-3}$ mutations/bp × generation (Maizels, 1995; Wabl et al., 1987), which is $\sim 10^{6}$ higher than the spontaneous background mutation rate of a transcribed housekeeping gene such as HPRT (Sale and Neuberger, 1998). The findings that EBV induces expression of AID mRNA and that CSR takes place in freshly, EBV-transformed PBL (He et al., 2003), indicate that a functional AID protein is expressed in LCLs.

Expression of the AID mutator in LCLs raises the possibility that it may also be responsible for the observed instability and frequent initial loss of antibody activity in antibody-producing LCLs (Glasky and Reading, 1989; Kozbor and Roder, 1981; Roder et al., 1986).

The aim of the present work was to determine whether transformation with EBV of peripheral blood B cells induces somatic mutations in the IgV region genes. Our findings show that somatic mutations are indeed generated in an antibody-secreting LCL and that it could lead to the loss of antigen-binding activity.

2. Materials and methods

2.1. Cell lines and cloning

The LCL1, LCL2 and CBL-78 cell lines were generated in the laboratory of Dr. H. Ben-Bassat and CBL-LS1 and the RF-AN cell lines were established in our laboratory (Steinitz and Tamir, 1982). The FLEB 14 lines 3 and 4 are pro-B cell lines generated by EBV transformation of embryonic liver lymphocytes (Altiok et al., 1989), and are designated LCL-EL. All of the cell lines were maintained in RPMI 1640 medium supplemented with 10% FCS, 2 mM glutamine, 100 μ/ml penicillin and 100 μg/ml streptomycin. Limiting dilution subcloning was performed using 96-micro-well plates and seeding 0.5–10 cells/well in 200 μl medium/well together with $(1–2) \times 10^{5}$ cells/well β-irradiated (2500 Rad) peripheral blood lymphocytes (PBL), as feeder cells. The medium contained 1 μg/ml of cyclosporine A. Clones were isolated from plates in which less than 30% of the wells showed cell growth. Wells which contained only irradiated feeder cells did not show any cell growth.

2.2. ELISA

The levels of the mu and lambda Ig chains secreted by the RF cells were determined using ELISA as described (Steinitz et al., 1988). Briefly, 96-microwell plates were pre-coated overnight at 4 °C with 50 μl/well of rabbit anti-mu or with rabbit anti-lambda (12 μg/ml, Dako), blocked by 250 μl of 0.5% gelatin in PBS and incubated with 50 μl/well duplicates of the cell culture supernatants. Following washing, 50 μl of diluted (1:20,000) alkaline phosphatase (AP)-conjugated goat anti-human immunoglobulin (Sigma) was added for 1 h at 37 °C. The color reaction was developed with substrate S104 (Sigma) and read in an ELISA reader at 405 nm. The antibody binding-activity of the rheumatoid factor produced by the RF cell lines was assessed by ELISA. Microwells were pre-coated with rabbit IgG (50 μl of 2 μg/ml) and then blocked as above. Cell supernatants were added and following incubation and washing, AP-conjugated goat anti-human mu antibody (Sigma) was added and the color reaction was developed and read as above.

2.3. Hermagglutination and rossete forming cells (RFC)

The ability of the IgM secreted by the RF cultured cell to bind IgG was examined by hemagglutination assay using human IgG-coated erythrocytes (Steinitz and Tamir, 1982). The plain culture medium and diluted serum from a rheumatoid arthritis patient served as negative and positive controls, respectively.

The binding specificity of the RF cells was examined by RFC assay using IgG-coated-erythrocytes. One hundred thousand lymphocytes mixed at 4°C with 10-fold excess of erythrocytes, pelleted and delicately resuspended and observed under light microscopy (Steinitz et al., 1977; Steinitz and Tamir, 1982).

2.4. EBV infection

Infection of PBL was performed essentially as described (Geylis et al., 2005) omitting the pre-selection step for specific antibody-expressing cells. Briefly, 10^{7}–10^{8} lymphocytes separated by Ficoll-Hypaque from blood were incubated for 1 h at 37 °C with 1 ml of the supernatant derived from the EBV-secreting B95-8 cell line. The cells were then seeded into 50 ml culture bottles at 2×10^{6} cells/ml in RPMI 1640 containing FCS and antibiotics as above in the presence of 1 μg/ml of cyclosporine A and incubated in a CO_2 incubator. Cell growth was recorded 4–5 weeks post-infection.

2.5. Nucleic acid purification

Genomic DNA was purified from $(5–10) \times 10^{6}$ cells by the SDS-proteinase K method (Maniatis et al., 1989). Total cellular RNA was isolated according to the manufacturer's instructions from $\sim 5 \times 10^{6}$ blood or cultured cells by the "High pure RNA isolation kit "(Roche, Mannheim, Germany), which includes a DNase I digestion step.