Virus-Cell Interactions in a Natural Killer-Like Cell Line From a Patient With Lymphoblastic Lymphoma

By Hirokazu Kanegane, Frederick Wang, and Giovanna Tosato

Lymphoproliferative disorders involving Epstein-Barr virus (EBV) infected natural killer (NK) cells are reported with increasing frequency, but the nature and role of EBV infection in these cells remains undefined. In this study, we have investigated virus-cell interactions in the EBV-positive YTnlO cell line, an NK-like cell line established from a patient with lymphoblastic lymphoma. Low level expression of the EBV receptor CD21 molecule was detected by FACS and reverse transcriptase polymerase chain reaction (RT-PCR) analysis. Immunoblotting and RT-PCR analysis identified a latency II pattern of EBV gene expression, consisting of EBNA-1 transcription from the Qp promoter, in the absence of other EBNA gene expression, and accompanied by LMP-1 and LMP-2A expression. The EBV genome was present in episomal form and there was evidence for lytic viral replication. This latency pattern is typical of EBV gene expression in nasopharyngeal carcinoma and Hodgkin’s disease, and differs from the full spectrum of EBV latent gene expression in most posttransplant lymphoproliferative disorders and from the restricted EBNA-1 expression in Burkitt’s lymphoma tissues. The interaction between EBV and NK cells described here has important implications for the pathogenesis and treatment of EBV-infected NK malignancies.

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MATERIAL AND METHODS

Cell lines. YTnlO cells, a subclone of the YT-cell line that was established from the pericardial fluid of a 15-year-old boy diagnosed with lymphoblastic lymphoma,27,28 was selected from the YT-cell line on the basis of its high cytotoxic activity in Dr J. Yodoi’s laboratory (a kind gift of Drs A. Yamauchi and J. Yodoi, Kyoto University, Kyoto, Japan). The cell lines B95-8, VDS-O, Ag876, and BJAB were used as representatives of an EBV-producer cell line, LCL, EBV-positive Burkitt’s lymphoma, and EBV-negative B-cell line, respectively.7 All cell lines were mycoplasma-negative, and were maintained with RPMI 1640 medium (GIBCO BRL, Grand Island, NY) supplemented with 10% heat-inactivated fetal calf serum (FCS; Intergen, Purchase, NY), 2 mMol/L L-glutamine (GIBCO BRL), and 5 µg/ml of gentamicin (Sigma Chemical, St Louis, MO).

Monoclonal antibodies (MoAbs) and immunofluorescence analysis. Fluorescein isothiocyanate (FITC)-conjugated MoAb against CD3 (Leu-4), and phycoerythrin (PE)-conjugated MoAbs against CD16 (Leu-11b), CD20 (Leu-16), CD23 (Leu-20), CD56 (Leu-19), and HLA-DR were purchased from Becton Dickinson Immunocytometry Systems (San Jose, CA). Anti-CDD1 (anti-CR2) MoAb and anti-HLA DR MoAb were raised against purified HLA-DR and CCR2, respectively. Anti-LMP-2A MoAb was a gift from Dr P. Cleary (Everest, MD) and was raised against the recombinant form of LMP-2A.

Expression of the full array of EBNA and LMP genes, particularly of the growth-promoting LMP-1 gene, may reflect the need of EBV to rapidly colonize the host by driving the proliferation of virally infected B cells. By contrast, limited expression of EBV genes, namely of EBNA-1 and of LMP-2A proteins that are devoid of growth-transforming properties per se, may reflect a strategy for virus survival in a resting B cell, mostly avoiding immune recognition.

streptavidin-PE were also obtained from Becton Dickinson. Biotinylated MoAbs against CD45RA and CD45RO were purchased from Pharmingen (San Diego, CA) and Dako (Carpenteria, CA), respectively.

The MoAbs anti- Tac (anti-interleukin [IL]-2Ra, mouse immunoglobulin G2a [IgG2a]) and anti- LMP-I (mouse IgG1) were kind gifts from Dr. R. H. D. Beard (National Institutes of Health, Bethesda, MD); TUG#1 (anti-IL-2Gy, rat IgG2b) was a kind gift from Dr. K. Sugamura (Tohoku University, Sendai, Japan); and the anti-early antigen-diffuse component (EA-D) MoAb R3 (mouse IgG1) was a kind gift of Dr. G. Pearson (Georgetown University, Washington, DC). PE-conjugated goat antimouse IgG1 and antirat Ig were obtained from Southern Biotechnology Associates (Birmingham, AL). FITC-conjugated goat antimouse IgG antibody was obtained from Cappel, Organon Technical (Durham, NC). Immunofluorescence was performed by direct or indirect methods, and the stained cells were analyzed using FACScan flow cytometer equipped with a Cell Quest data analysis program (Becton Dickinson), as described previously.37 For analysis of EA-D expression, fluorescence microscopy was used.

**Immunoblotting.** Cells were washed twice in phosphate-buffered saline (PBS), and cell pellets were lysed in electrophoresis sample buffer and boiled for 10 minutes. The equivalent of 2 × 106 cells was loaded into each lane of 8% Tris-glycine gels (Novex, San Diego, CA). The electrophoresed proteins were transferred onto nitrocellulose membranes (Immobilon-P; Millipore, Bedford, MA). Blots were washed twice in PBS with 0.05% Tween 20 (PBS-T), blocked in 5% skim milk in PBS overnight, and incubated with human serum with high titer antibodies against EBNAs, with a MoAb directed against LMP-I (S12, mouse IgG2a),38 with a pool of MoAbs directed against EBNA-2 (See Table 1), or with a MoAb directed against EBNA-2 (PE2, mouse IgG1)39 in PBS-T at room temperature for 1 hour. After rinsing four times in PBS-T for 5 minutes, the blots were incubated for 1 hour either with horseradish peroxidase-conjugated protein A (Bio-Rad Laboratories, Hercules, CA) for detection of bound antibody in human serum and in the S12 (anti-LMP-1) MoAb, or with horseradish peroxidase–conjugated goat antimouse IgG1 (Southern Biotechnology Associates) for detection of bound CS1-4 (anti-LMP-I) MoAb and bound EBNA-2–specific MoAb. Immunoblots were rinsed five times for 10 minutes in PBS-T, and then developed by using the ECL Western blotting detection systems (Amersham Life Science, Arlington Heights, IL). Prestained molecular weight markers (rainbow-colored protein) were obtained from Amersham.

**DNA preparation and polymerase chain reaction for EBV DNA.** Cells were washed twice in PBS and cellular DNA was extracted with phenol and chloroform, precipitated with ethanol, and resuspended in distilled water. A 0.1-µg quantity of each sample was used for one-step typing of EBV by polymerase chain reaction (PCR) amplification as described elsewhere.31 The oligonucleotide sequences for amplification of the EBV U2 region were 5'-TTTCACTCAATACATGAAACC-3' (2771-2789) and 5'-TGCCCAAAAGTCGTGAAACAC-3' (3149-3130). An amplification was performed in 50 µL containing 20 mmol/L Tris-HCl (pH 8.4), 50 mmol/L KCl, 1.5 mmol/L MgCl2, 0.2 mmol/L dNTP mixture, 0.4 µmol/L of each primer, and 2.5 U of Taq DNA polymerase (GIBCO BRL). An initial denaturation step of 3 minutes at 95°C was followed by 35 cycles of denaturation at 95°C for 1 minute, annealing at 57°C for 1 minute, and extension at 72°C for 2 minutes followed by a final extension of 7 minutes at 72°C. The DNA separated through the gel was partially depurinated with sodium bisulfite, hybridized to an EBV BamHI DNA fragment labeled with 32P by random priming. The filter was washed with 0.1× saline sodium phosphate buffer (pH 8.0) and exposed to X-ray film.
EBV LATENT GENE EXPRESSION IN AN NK-LIKE CELL LINE

Table 1. Oligonucleotide Primers Used in RT-PCR Analysis

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<th>Name</th>
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<td>U-K (Gp/Wo-initiated)</td>
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<tr>
<td>3'</td>
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* Number indicates 5' codon corresponding to the EBV B95-8 genome.

citrate (SSC), 1% SDS, and exposed to Kodak XAR film (Eastman Kodak, Rochester, NY) at -70°C.

RESULTS

The cell-surface phenotype of YTN10 cells was analyzed to confirm that they had maintained their originally described NK phenotype and to determine if they expressed any cell marker typical of EBV infection.2,28 YTN10 cells expressed the NK-cell–specific surface determinant CD56, as well as HLA-DR antigens, but did not express other NK-cell–associated marker CD16 antigen or the mature T-cell–associated marker, CD3 antigen (Fig 1).27,28 Whereas the original YT cells expressed CD25, the IL-2R alpha chain,27 the YTN10 subclone did not express this antigen. As previously reported for the parental YT cells and characteristic for NK cells,41 YTN10 cells expressed the IL-2R beta and gamma chains at high levels. The CD45RO antigen was also highly expressed on YTN10 cells, but the CD45RA antigen was only weakly expressed. Although it was reported that the B-cell–associated markers CD20, CD21, and CD23 antigens were absent on YT cells,28 we found them to be expressed at very low levels on YTN10 cells. Thus, YTN10 cells belong to the NK-cell lineage, as they express surface markers characteristic of NK cells, and fail to express or express at very low levels T-cell– and B-cell–associated markers.

The CD21 antigen is the EBV receptor on B lymphocytes,22 and CD21 transcripts are present in epithelial cells that can also be infected with EBV.2,3,28 We looked for EBV receptor transcripts by reverse transcriptase (RT)-PCR analysis (Fig 2). The expected 497-bp band was detected in the sample from the lymphoblastoid VDS-O cell line. In addition, a faint 497-bp band was reproducibly derived from YTN10 cells (Fig 2). These results indicated that YTN10 cells express the CD21 gene, and suggest that EBV may utilize a common cell-surface receptor to infect B cells, epithelial cells, and NK cells.

In situ hybridization using probes for the highly transcribed EBER showed that greater than 95% of YTN10 cells are EBER-positive, although the signal was weaker than that derived from B95-8 and VDS-O cells used as positive controls (Fig 3). The EBV-negative BJAB cells gave negative results. Expression of EBV latent genes in YTN10 cells was first examined by Western blotting. Using a human serum reactive to EBNA's,22 YTN10 cells were found to express EBNA-1, but not EBNA-2 (Fig 4A). Differences in the EBNA-1 molecular weight are common due to viral strain variation of the internal repeat region 3 within the EBNA-1 coding region.1 The EBNA-1 in YTN10 cells, approximately 85 Kd, was slightly smaller than the EBNA-1 in B95-8, VDS-O, and Ag876 cells (approximately 87 Kd), indicating that the EBV strain infecting YTN10 cells was distinct from those common laboratory strains. EBV early-antigen expression was also detected in YTN10 cells, indicating that a fraction of the cells were undergoing lytic EBV infection (Fig 4A).

The absence of EBNA-2 expression in YTN10 cells could not be attributed to infection with a type 2 (or B) EBV strain. Two types of EBV have been classically defined by differences in the U2 region encoding EBNA-2 resulting in distinct serologic reactivities.41 PCR amplification with EBNA-2–specific primers, which distinguish type 1 (or A) and type 2 (or B) EBNA-2, indicated that YTN10 cells are infected with a type 1 (or A) EBV (Fig 5). As expected, type 1 EBV was detected in B95-8 and VDS-O cells, whereas type 2 EBV was detected in Ag876 cells (Fig 5). In addition, immunoblotting with a MoAb directed to a type-independent epitope of EBNA-2 detected both the EBNA-2A in B95-8 and VDS-O cells and the EBNA-2B in Ag876 cells, but failed to detect either EBNA-2 protein in YTN10 cells (Fig 4D).

EBNA-1 expression in the absence of other EBNA's is consistent with a latency I or II pattern of viral gene expression that is typically associated with Burkitt's lymphoma tumors, nasopharyngeal carcinomas, and Hodgkin's disease.18-21 In latency I and II, EBNA-1 is transcribed from a
unique promoter approximately 50 kb downstream of the EBNA promoter used in latency III. These latency patterns can be distinguished in RT-PCR assays using either primers that specifically identify the latency I/II EBNA-1 transcripts originating from a promoter (Qp) in the BamHI Q region of the viral genome, or primers that identify latency III EBNA-1 transcripts originating from either the BamHI C promoter (Cp) or the BamHI W promoter (Wp). As shown in Fig 6, EBNA-1 transcripts could be RT-PCR-amplified from all EBV-infected cells, including YTN10 cell using primers in the common EBNA-1 BamHI U and K exons. In contrast, no EBNA-1 transcripts were derived from the EBV-negative BJAB cell line. EBNA-1 transcripts typical for latency III (EBNA-1 transcribed from either the Cp or Wp promoter detected with primers localized in the exons BamHI K and BamHI Y3) could be derived from B95-8, VDS-O, and Ag876 cells, but not from YTN10 cells (Fig 6). As expected, a percentage of B95-8 and Ag876 cells also expressed latency I/II EBNA-1 transcripts (EBNA-1 transcribed from the Qp promoter detected with primers from the BamHI Q and BamHI K exons). EBNA-1 transcripts in YTN10 cells could only be detected with primers from the BamHI Q and BamHI K region of the viral genome, consistent with a latency I/II transcription pattern initiated from the promoter Qp (Fig 6). EBNA-2 transcripts could not be amplified from YTN10 cells (Fig 6), consistent with the immunoblotting classification of YTN10 cells in latency I/II. In control experiments, EBNA-2 transcripts were amplified from the type 1 EBV-
positive B95-8 and VDS-O cell lines, but not from the type 2 EBV-positive Ag876 cell line and the EBV-negative BJAB cell line.

Latency I and II are distinguished by expression of most of the LMP genes in latency II but not in latency I. A strong LMP-1 transcript was detected in YTN10 cells by RT-PCR analysis using primers from the 5’ end of the LMP-1 gene that distinguish a latent LMP-1 transcript from a late lytic transcript sharing the 3’ end of the LMP-1 exon. LMP-1 transcripts were also derived from the EBV-positive B95-8, VDS-O, and Ag876 cells, but not from the EBV-negative BJAB cell line. Immunoblotting showed that LMP-1 molecular size in Ag876 cells was smaller than B95-8 and VDS-O cells (Fig 4B), and these differences might be due to different sequences in LMP-1 carboxy termini. Failure of the S12 MoAb to detect an LMP-1 gene product from YTN10 cells on Western blotting (Fig 4B) may be due either to EBV strain variation in the LMP-1 carboxy terminus where the S12 epitope is located, or to low-level LMP-1 expression not detectable by Western blotting. To examine these possibilities, we used a pool of CS1-4 MoAbs that recognize epitopes distinct from that recognized by the S12 MoAb. As shown in Fig 4C, CS1-4 MoAbs detected an LMP-1 protein from YTN10 cells. In addition, immunostaining of YTN10 cells using CS1-4 MoAbs detected 23% cell positivity, whereas the S12 MoAb consistently gave negative results (data not shown). Sensitive antisera for LMP-2 expression are not readily available, so that LMP-2 expression is typically assayed by RT-PCR. LMP-2A-specific primers consistently detected a weak but positive LMP-2A transcript in YTN10 cells (Fig 6). LMP-2A-specific transcripts were also derived from the B95-8, VDS-O, and Ag876 cells, but not from the EBV-negative BJAB cell line. Thus, RT-PCR analysis is typical for latency II viral gene expression in YTN10 cells.

Restricted EBV gene expression could also be due to abnormal integration and disruption of latent gene coding sequence. Analysis for episomal EBV DNA by in situ lysing gels, as described by Gardella et al, detected the presence of an EBV episome similar in size to that present in B95-8 and VDS-O cells, suggesting the presence of an intact EBV genome in YTN10 cells (Fig 7). This analysis also indicated a relatively low viral copy number in YTN10 cells. Presence of a faster migrating band in YTN10 cells is consistent with linear viral DNA and viral replication (Fig 7), as suggested by the immunoblot detection of early antigen lytic cycle gene expression (Fig 4A). In addition, immunofluorescence analysis of YTN10 cells using the anti–early antigen MoAb R3 detected a few (0.2%) clearly positive cells (data not shown).

**DISCUSSION**

EBV has developed a successful strategy for infection, spreading, and persistence in humans that uses a dynamic
range of viral gene expression. Key features to the successful penetration of EBV in humans are the intermittent viral replication in the pharyngeal epithelium that ensures spread to new hosts, and the establishment of a lifelong latency in B cells that ensures viral persistence. B lymphocytes appear central to EBV persistence in humans. During initial EBV infection associated with IM, a relatively large pool of latently infected B cells is generated. Recently, it was reported that the spectrum of viral RNAs expressed in these cells is broad, including all six nuclear antigens (EBNA-1, -2, -3A, -3B, -3C, -LP) and the membrane proteins (LMP-1 and -2A). This pattern is comparable to that found in B-lymphocyte growth immortalized by EBV in vitro, and was interpreted to reflect the need of EBV to rapidly colonize the host, before the development of specific immunity. Once the host-cell–mediated responses develop, the pool of EBV-infected B cells in the circulation is substantially reduced, and is believed to reside in nonproliferating cells. It was reported that the spectrum of viral RNAs expressed in these cells is limited to EBNA-1 and LMP-2A, viral products not known to have growth-transforming ability. This more restricted form of latent EBV gene expression may reflect the need of EBV to persist in the host without destroying cytotoxic T cells.

EBV latent gene expression is equally diverse in various EBV-associated malignancies. EBV-positive Burkitt’s lymphomas generally express only EBNA-1 and low levels of LMP-2A, a pattern believed to be shared by most latently infected normal peripheral blood B cells in sera positive for EBV. However, unlike these normal, phenotypically resting cells, Burkitt’s cells are cycling and rapidly expanding. It was suggested that this proliferative state is not driven by EBV, but by the constitutive expression of c-myc secondary to its translocation. Thus, EBV would be maintained in the dividing tumor cells by virtue of EBNA-1 expression, but would not play a major role in the expansion of the malignant cells. In contrast, most EBV-infected B cells in posttransplant lymphoproliferative disease typically express the latency III program, which probably reflects the direct dependence of these tumors on the growth-promoting effects of the EBV latent genes. Yet another pattern of EBV latency was described in EBV-positive nasopharyngeal carcinomas, Hodgkin’s disease, and T-cell lymphomas. Here EBNA-1 is expressed in the absence of other nuclear proteins along with variable amounts of LMP-1 and -2 ranging from trace levels to high levels. This pattern of EBV latency, recently described also in epithelial cells infected with EBV, may represent a compromise by the virus to fulfill the same time both a need to promote growth and to limit immune recognition.

Despite the fact that EBV-associated lymphoproliferative diseases with an NK-like phenotype are reported with in-

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**Fig 4.** Western blot analysis of EBV gene expression in YTN10 cells. Cell extracts from 2 x 10⁶ cell equivalents were electrophoresed through 8% Tris-glycine gels, transferred onto nitrocellulose membranes, probed with appropriate antibodies, and developed. (A) Membrane probed with a human serum containing high-titer antibodies to EBV. (B) Membrane probed with a murine MoAb to LMP-1 (S12). (C) Membrane probed with a murine MoAb to LMP-1 (CS1-4). (D) Membrane probed with a murine MoAb to EBNA-2 (PE2).

**Fig 5.** EBV typing of virus-infected cell lines. DNA was extracted from the cells and subjected to PCR amplification for EBNA-2 (U2 region) with appropriate primers designed to discriminate between type 1 and type 2 EBV. The amplified products were electrophoresed through a 2% agarose gel.
Increasing frequency, little is presently known about virus-cell interactions in these cells. In addition, lymphoproliferative diseases involving EBV-infected NK cells include acute and rapidly fatal leukemias, nodal and extranodal lymphomas, progressive lymphoproliferative disorders, and, occasionally, chronic benign expansions of NK cells. It is conceivable that virus-cell interactions might differ in the various disorders, and that these differences might underscore different roles that the virus plays in the disease pathogenesis.

In the present studies, we have shown that the NK-like YT10 EBV-positive cell line derived from a patient with the diagnosis of lymphoblastic lymphoma is infected with type I EBV, contains both episomal and linear DNA, expresses EBV receptor molecules albeit at low levels, and exhibits a type II virus latency program characterized by expression of EBNA-1 in the absence of other nuclear proteins, accompanied by low level expression of LMP-1 and LMP-2A. As described here, this latency pattern is observed in the majority of nasopharyngeal carcinomas, Hodgkin’s disease, and T-cell lymphomas, and contrasts with the latency III pattern typically observed in EBV-positive B cells in posttransplant lymphoproliferative diseases. The distinction between latency I and II can be somewhat arbitrary, because the presence of weak LMP transcripts may be due to a fraction of latency I cells making a transition to latency II, as shown by certain Burkitt’s tumor cell lines grown in tissue culture. However, in contrast to these Burkitt’s tumor cell lines cultured in vitro, the latency II pattern in YT10 cells is noticeable for its stability and complete lack of latency III expression over time. Thus, the EBV latency in this lymphocytic cell line of NK lineage is more reminiscent of the tight latency II pattern in epithelial cells and Hodgkin’s disease.

The ability of YTN10 cells to proliferate in the absence of growth-promoting latency I EBV gene expression suggests that EBV infection is not likely to be the sole reason for their immortalized state. Although the transforming/immortalizing event(s) remain undefined, the observation that YTN10 cells have an abnormal karyotype, namely are tetraploid and exhibit a 4+ duplication, is consistent with the possibility that the cells have incurred some genetic damage in addition to EBV infection. EBV was found to be monoclonal on early passage YT cells, suggesting that EBV infection preceded and possibly contributed to the malignant transforming event, similar to the viral pathogenesis implicated in Burkitt’s lymphoma and nasopharyngeal carcinoma.

The expression of early antigen complex-associated proteins and the existence of linear EBV forms in YTN10 cells...
indicate the occurrence of virus production. Previous studies have identified a distinct in vitro pathway of direct entry of cells with lytic virus cycle without associated broadening of the pattern of latent nuclear antigens expression. When activated with anti-Ig, Akata cells were found to continue expression of EBNA-1 from the Qp promoter, and to rapidly begin expression of LMP-1, -2A, and -2B together with early lytic cycle antigens. This pattern of gene expression is very similar to the pattern observed in YT1N0 cells. Activation from latency II/II directly into the cell cycle would represent a mechanism to avoid T-cell immunosurveillance. In addition, latency II/II cells could provide a reservoir for virus persistence and virus production.

The presence of EBV in malignant cells might be considered in the design of strategies for these disorders. We know that immunocompetent T cells are effective in reducing the pool of latently infected B cells. This is illustrated by the effectiveness of immune surveillance against EBV in normal seropositive individuals and by recent successes in the use of immune T cells for the treatment of EBV-positive B-cell lymphoproliferative diseases in immunosuppressed individuals. We would propose that use of therapeutic agents capable of eliciting expression of the full array of EBV latency genes in cells exhibiting a type I or type II latency program might represent an effective treatment strategy in immunocompetent individuals. Using this approach, one might be able to change a tumor cell from an immunologically invisible target to one fully recognized by immune T cells. The observation that Burkitt's lymphoma cells can be induced to switch from a type I to a type III latency by treatment with 5-azacytidine suggests that this approach can be entertained.

Although the present studies are limited to one NK-like cell line, they provide initial important information on the relationship between EBV and host cells with NK phenotype. Future studies will be necessary to extend these observations to include tissue specimens representative of the full spectrum of NK cell, EBV-positive lymphoproliferative diseases.

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H Kanegane, F Wang and G Tosato