Epstein-Barr Viral DNA in Acute Large Granular Lymphocyte (Natural Killer) Leukemic Cells

By D.N.J. Hart, B.W. Baker, M.J. Inglis, J.C. Nimmo, G.C. Starling, E. Deacon, M. Rowe, and M.E.J. Beard

Serologic studies in a male Caucasian presenting with an acute hepatitis-like illness, associated with an increase in peripheral blood large granular lymphocytes (LGLs), suggested a chronic or reactivated Epstein-Barr virus (EBV) infection. The LGL were shown to have a natural killer (NK) cell, CD3- CD16- CD56+ CD57- phenotype and mediated strong nonspecific major histocompatibility complex-unrestricted (NK) cytotoxic activity. A progressive increase in the peripheral blood LGL count was associated with a rapid deterioration, hepatic necrosis, and death. Widespread organ infiltration with LGLs suggested a malignant lymphoproliferative condition, but no lymphoid (T-cell receptor or IgH) gene rearrangement or cytogenetic marker was detected. However, molecular analysis identified EBV genomic DNA present in a single episomal form within the LGL, establishing the clonal nature of the LGL proliferation. Confirmation that the EBV had infected the leukemic LGL was obtained by in situ hybridization studies that showed EBV RNA within the LGLs. Immunoblotting of LGL protein extracts established that, of the EBV gene products, EBV nuclear antigen-1 (EBNA-1) was expressed but EBNA-2 and the latent membrane protein (LMP-1) were not detectable in the leukemic cells. These results suggest that EBV may be involved directly in LGL cell transformation, in a manner similar to EBV-associated B-cell lymphomas, although other molecular changes probably contribute to the evolution of a fully malignant leukemic clone.

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NATURAL KILLER (NK) cells, which mediate non-major histocompatibility complex (MHC)-restricted lysis of tumor and virus-infected cells without prior sensitization, are found in the 10% to 15% of peripheral blood lymphocytes that have large granular lymphocyte (LGL) morphology. Chronic increases in peripheral blood (PB) LGL counts have been described, typically associated with splenomegaly and neutropenia or other cytopenias. Surface phenotyping of these LGLs has confirmed that they often express one or more of the NK cell-associated leucocyte differentiation antigens CD16, CD56, or CD57. Although the majority of these expanded LGL populations also express a functional CD3 antigen and clonally rearranged T-cell receptor (TCR) complex, those LGLs that have cytotoxic activity against NK targets mediate this NK-like activity (NK cells by definition lack CD3/TCR) independently of the CD3/TCR complex. An indolent clinical course is usual and spontaneous regression has been observed in some cases. Serologic studies have raised the possibility of an associated human T-cell leukemia virus-1 (HTLV-I)-like retrovirus infection, but no molecular evidence to support this suggestion has been reported to date.

Infection with the Epstein-Barr virus (EBV) is associated with both a cytotoxic T-cell response directed against EBV-infected B lymphocytes and a transient increase in LGLs with enhanced NK activity. EBV may be associated with some sustained increases of LGLs has been raised recently. Acute syndromes in which the rapid proliferation of LGLs results in a clinical picture more closely resembling an acute leukemia or lymphoma are less common but have been described after a documented EBV infection. We recently observed a patient who died soon after presenting with an acute hepatitis and a rapidly escalating LGL count. Evidence was obtained to show that this acute leukemic process involved a clonal proliferation of abnormal NK cells containing EBV DNA.

CASE REPORT

A 50-year-old white man was admitted acutely in November 1988 with a 6-week history of lethargy and anorexia, followed by 3 days of abdominal fullness, fever, and jaundice. Examination showed a tender smoothly enlarged liver and a palpable spleen, but no peripheral lymphadenopathy. The blood count (Coulter Plus IV; Coulter, Hialeah, FL) showed a hemoglobin count of 178 g/L, platelets 37 × 10^11/L, and a white blood count (WBC) of 6.6 × 10^9/L, with 1.1 × 10^9/L LGL. Heterophile antibody tests were repeatedly negative. The biochemistry profile included a serum creatinine of 0.12 mmol/L, urea 4.2 mmol/L, albumin 37 g/L, bilirubin 262 μmol/L, conjugated bilirubin 204 μmol/L, alkaline phosphatase 485 U/L, γ-glutamyl transpeptidase 319 U/L, and aspartate aminotransferase 290 U/L. Protein electrophoresis showed an inflammatory response with normal Ig levels. The autoantibody screen was negative. The coagulation profile included an international normalized ratio of 1.3, activated partial thromboplastin time 27 seconds, and fibrinogen level 1.8 g/L. Ultrasound of the abdomen confirmed hepatosplenomegaly with no focal lesions and Doppler studies showed normal hepatic vein flow. No organisms were grown from blood cultures and hepatitis B surface antigen was not detected. No malarial parasites were seen. Serologic testing detected no evidence of recent infection with Brucella, Herpes Simplex virus, Cytomegalovirus, Toxoplasma, Leptospira, Hepatitis A, syphilis, HTLV-I, or human immunodeficiency virus 1 (HIV-1). The EBV serology is summarized in Table 1, and is suggestive of chronic or reactivated infection. An initial diagnosis of a viral hepatitis, possibly secondary to EBV, was made. The patient deteriorated over the subsequent 2 weeks with fluctuating fever and increasing bilirubin, liver enzyme, and creatinine levels accompanying a marked increase in LGLs (WBC, 53 × 10^9/L; LGL, 44). A computed tomography (CT) scan showed enlarged para-aortic nodes in addition to hepatosplenomegaly. Bone marrow aspirate showed infiltration by a population of LGLs.

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Table 1. EBV Serology

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<th>Antigen</th>
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<th>Titer</th>
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<tr>
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<td>IgM</td>
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comprising 61% of nucleated marrow cells. The trephine appearances were of a diffuse mononuclear cell infiltration, most prominent in the paratrabecular areas (Fig 1B) with a patchy increase in reticulin. Cytogenetic analysis of marrow cells was normal. The immunophenotypic analysis of peripheral blood and bone marrow was suggestive of an abnormal NK cell proliferation (Table 2).

Fifteen days after admission, methylprednisolone 40 mg intravenously for 6 hours was commenced, but other cytotoxic drugs were

Fig 1. Cytologic and histologic features of the LGL leukemia. (A) Peripheral blood LGLs. (MGG, original magnification x1,000.) (B) Bone marrow trephine showing prominent mononuclear cell infiltration in a paratrabecular area. (H and E, original magnification x200.) (C) Postmortem section of liver with diffuse mononuclear cell infiltrate and destruction of normal hepatic architecture. A few hepatocytes remain. (Hematoxylin and eosin [H and E], original magnification x400.) (D) Transmission EM of peripheral blood LGLs. (Original magnification x21,500.)
were stained with May-Grunwald-Giemsa (MGG), Sudan Black, and mononuclear cells were harvested. The monoclonal antibodies (MoAbs) used to phenotype this patient’s mononuclear cells by immunofluorescence and flow cytometry on an EPICS Profile (Coulter) are detailed in Table 2. Fluorescein isothiocyanate-goat antimouse (FITC-GAM; Tago, Burlingame, CA) was used as the second antibody to label antibody-reactive cells.

Immunophenotypic analysis of postmortem tissue specimens were performed using an immunoperoxidase technique as previously described.16

Cytotoxicity assays. Cytotoxicity against the erythroleukemia cell line, KG62, was determined using a standard 31Cr release assay in 96-well microtitre plates (Flow Laboratories, McLean, VA).

Antibody-dependent cellular cytotoxicity (ADCC) assays were performed using 31Cr-labeled group O Rh-positive erythrocytes labeled with a mix of the MoAbs CMRF-4 (IgG3), CMRF-10 (IgG3), and CMRF-17 (IgM), which react with different erythrocyte antigens.17 Normal peripheral blood mononuclear cells (PBMC) were used as a positive control in both assays.

DNA analysis. Genomic DNA was extracted from PBMC according to standard methods. DNA was also extracted from CD19+ PBMC that were obtained by separation using the MoAb FMC63 and M-450 Dynabeads (Dynal, Oslo, Norway) according to the manufacturer’s instructions. Purified DNA (11 µg) was digested with restriction endonucleases as per the manufacturer’s instructions (New England Biolabs, Beverley, MA) and hybridized at 65°C followed by washing at appropriate stringency. TCR-γ chain gene rearrangements were tested using oligonucleotides and polymerase chain reaction (PCR) conditions as described by Trainor et al.18 Two sets of HTLV-1 envelope primers were used to perform PCR with specific oligonucleotide probing of the products as detailed by Daenke et al.19 Positive controls for EBV included the B95-8 and Raji cell lines20 and the EBV-transformed lymphoblastoid cell line X50-7. Positive control HTLV-1 DNA was provided by Dr. C.R.M. B bangham (Oxford University, UK).

In situ hybridization. In situ RNA-RNA hybridization was performed as described in detail by Niedobitek et al.21 The plasmids pBslJ1 and pBslJ2,21 containing the EBV-encoded small, nonpolyadenylated RNAs (EBER)-1 and EBER-2-specific fragments,22 were used to prepare 35S-labeled sense and antisense probes. The size of the probes were adjusted to about 100 to 200 bases and the two sense (pBslJ1 and pBslJ2) and two antisense probes mixed to produce combination sense and combination antisense probes of increased sensitivity. After the preparation of the tissue sections,21 the labeled probes were hybridized at 50°C for 12 hours. Excess probe was removed, then the slides were rinsed in 2X SSC, dipped in Ilford G5 emulsion, exposed, developed, and then counterstained.21

Immunoblotting of EBV proteins. Cell extracts from 106 cells (per track) were separated on Laemmli 7.5% discontinuous polyacrylamide gels, electroblotted onto nitrocellulose filters, and subsequently probed with selected human sera, rabbit antiserum, or mouse MoAbs followed by incubation with [125I] protein A as previously described.23 A polyspecific high titer human serum PBE was used for detection of EBV nuclear antigens (EBNAs)-1, -2, -3c, and -LP. The MoAbs PE2 and CS-1-4 were used to detect EBNA-2 and latent membrane protein-1 (LMP-1), respectively.24

### Table 2. Surface Marker Characteristics

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Abbreviations: ATCC, American Type Culture Collection (Rockville, MD); Ortho, Ortho Pharmaceutical Corp (Raritan, NJ); BD, Beckton Dickinson (Mountain View, CA); 4th LDAH, 4th Leucocyte Differentiation Antigen Workshop; ND, not done; PM, postmortem; WP, weak positive.
RESULTS

Characterization of LGLs. The peripheral blood and bone marrow LGLs were irregularly shaped and of variable size with oval or slightly indented nuclei and relatively clumped chromatin. Large azurophilic granules were prominent within the abundant pale blue cytoplasm of MGG-stained cells (Fig 1A). Granular staining was noted for acid phosphatase and nonspecific esterase (fluoride sensitive), but the Sudan Black, peroxidase, and periodic acid Schiff stains were negative.

Transmission EM of the LGLs showed a number of distinctive cytoplasmic features, including membrane-bound electron-dense and lucent granules, significant glycogen accumulations, fat droplets, membrane whorls, an active Golgi apparatus, and free ribosomes (Fig 1D). No virus particles were observed in any of the sections.

The phenotype of peripheral blood and bone marrow mononuclear cells was CD2+, CD3-, CD4+, CD8-, CD11b-, CD16-, CD56+, CD57- (Table 2). Analysis of postmortem samples of liver, spleen, and lymph nodes showed diffuse infiltration by mononuclear cells of the same phenotype.

Cytotoxicity assays. Cells from this patient lysed the NK target K562 at low effector target ratios and also lysed the normally NK-resistant MANN cell line. Low levels of ADCC were noted (Fig 2).

EBV serology. The EBV serology results are summarized in Table 1 and indicate chronic or reactivated EBV infection. Western blotting showed that the antibodies were reactive with EBNA-1, EBNA-2, and EBNA-3c, the leukemic LGLs were shown to express EBNA-1 but not EBNA-2 or EBNA-3c (Fig 5A). The absence of EBNA-2 in the cells was further confirmed by an identical immunoblot probed with MoAb PE-2 (Fig 5B). Likewise, blots probed with the MoAb CS1-4 showed that the leukemic cells did not express LMP-1. Immunofluorescence studies with the antibodies PE-2 and CS1-4 on tissue sections confirmed that EBNA-2 and LMP-1 were not expressed.

DNA analysis. Southern blot analysis showed the TCR-β chain and Ig genes to be in the germline configuration and no TCR-γ rearrangement was detected using PCR (not shown). The PCR amplification and specific oligonucleotide probing for HTLV-1 proviral DNA was negative (not shown). A single band of 8.7 kb was detected in the DNA prepared from PBMC that was cut with BamHI and probed with the Xho I 1.9 EBV probe to the unique DNA sequence adjacent to the right terminal repeat region of the EBV genome (Fig 3A). This result was confirmed using B-cell (CD19)-depleted PBMC, suggesting the EBV DNA was contained within the leukemic LGLs. After stripping the blot and reprobing, the same bands also hybridized with the EcoRI 1.1 EBV probe to the unique DNA adjacent to the left terminal repeat region of the linearized EBV genome. Double digestion with BamHI and Bgl II before blotting and hybridization with the Xho I 1.9 EBV probe showed two fragments of 5.8 kb and 2.5 kb (Fig 3B), confirming the presence of a clonal episomal form of EBV carrying three terminal repeat sequences within the joined termini of the viral genome.

In situ RNA-RNA hybridization. Direct evidence for the presence of EBV within the leukemic LGLs was sought by in situ hybridization. Sections of a lymph node that was virtually completely replaced with LGLs of the same phenotype as detailed in Table 1 were probed using a SRNA probes to EBER-1 and EBER-2. Labeling of the LGLs with the antisense probe but not with the control sense probe confirmed the presence of EBV-RNA for these EBV gene products within the leukemic cells (Fig 4).

EBV protein expression. Cell lysates prepared from blood leukemic LGLs were immunoblotted to identify EBV proteins. Using a polyclonal human antiserum reactive with EBNA-1, EBNA-2, and EBNA-3c, the leukemic LGLs were shown to express EBNA-1 but not EBNA-2 or EBNA-3c (Fig 5A). The absence of EBNA-2 in the cells was further confirmed by an identical immunoblot probed with the MoAb PE-2 (Fig 5B). Likewise, blots probed with the MoAb CS1-4 showed that the leukemic cells did not express LMP-1. Immunofluorescence studies with the antibodies PE-2 and CS1-4 on tissue sections confirmed that EBNA-2 and LMP-1 were not expressed.

DISCUSSION

Acute LGL proliferations such as that occurring in this patient are seen much less frequently than the chronic form of the disease. However, in marked contrast to the chronic LGL proliferations, the cells in this case and the other 14 acute LGL leukemias described have all been CD3+, and the TCR genes, when tested, were not rearranged. This, combined with the NK functional activity of the LGLs, is good evidence for classifying acute LGL leukemia as a disease of the NK-cell lineage. To date, the reports of aggressive disease involving CD3+ LGLs have been confined to lymphomas and, in the one case tested, TCR gene rearrangement was documented, a phenotype more typical of the majority of the chronic LGL proliferations. Although these CD3+/TCR-positive cells may have "NK function," there is no evidence to date that these two cell types can differentiate one to the other and they must, for the moment, be considered to be the product of two individual differentiation pathways.

This patient presented with a modest increase in LGLs, which was initially considered to be reactive, but the subsequent rapid escalation in circulating LGLs suggested a leukemic transformation had occurred. It has only been possible in a few instances of aggressive LGL proliferation to document a cytogenetic abnormality as evidence of clonality and, therefore, malignancy. No cytogenetic marker was found in this case, but clear evidence of the
clonal nature of the LGL proliferation was obtained by finding the single episomal form of EBV DNA present in the leukemic cells. This result reinforces the value of this approach for determining the clonality of EBV-associated proliferations that lack a chromosome abnormality. Confirmation that the leukemic LGLs had been infected with EBV was obtained by in situ hybridization.

The demonstration of both EBV DNA and RNA within the leukemic LGLs has implications for the possible pathogenesis of aggressive NK cell proliferations. Acute EBV infection may be associated with an atypical lymphocytosis that includes an increase in both NK cells and CD8+ cytotoxic T cells, as well as CD4+ cells. Although cytotoxic T cells are thought to play the major role in controlling the infection by killing EBV-infected B lymphocytes in an HLA-restricted cytotoxic T-cell response, it is clear that the infected B cells are also targets for NK effectors. A similar mild atypical lymphocytosis may persist in a minority of patients with serologic evidence of chronic EBV infection. There has been no evidence to date to suggest that the NK cells found in either acute or chronic EBV infections might harbor the EBV virus, but a recent report described EBV DNA within a transient polyclonal T-cell proliferation. The serologic features in

Fig 3. Molecular studies on the leukemic LGL DNA. (A) Autoradiograph of DNA from negative control, Raji (monoclonal Burkitt’s lymphoma) cell line, B95-8 cell line, and the patient, digested with BamHI and probed with the EBV Xho I 1.9 probe. The B95-8 cell line shows multiple bands, consistent with infection of a polyclonal line with different EBV genomes. The single bands seen in the Raji and the patient (8.7 kb) lanes are indicative of cellular monoclonality involving one EBV episomal form. The 8.7-kb band was also readily detected in DNA prepared from B-cell–depleted mononuclear cells (patient B-ve). (B) Double digestion of the patient’s LGL DNA with BamHI and Bgl II showed the 5.8- and 2.5-kb bands, after hybridization with the EBV Xho I 1.9 probe. HindIII-cut λ markers are shown.

Fig 4. In situ hybridization of leukemic LGLs using RNA probes (sense and antisense) to the EBV products EBER-1 and EBER-2. A section of lymph node confirmed by immunohistologic staining to be fully replaced by leukemic LGLs was probed in (A) with the antisense probe and in (B) with the sense probe.
Table 1 suggested a chronic or reactivated infection with EBV and it is interesting to speculate that the rapid increase in LGLs observed may have been preceded by a preleukemic phase, involving a polyclonal NK response, before the EBV leukemic clone became predominant.

EBV has been causally associated with malignant proliferations of B lymphocytes and epithelial cells of the nasopharynx for many years. The virus gains entry into B lymphocytes and epithelial cells of the pharynx via the CD21 molecule (C3d receptor), which acts as a receptor for the viral envelope protein. We were unable to detect significant expression of CD21 on the LGLs in this case, but it should be noted that CD21 is not detectable on some other EBV-positive malignancies, such as Burkitt's lymphoma and nasopharyngeal carcinoma. There are various explanations that might account for the presence of EBV DNA in cells lacking the specific EBV receptor. First, the level of receptor expression required for EBV entry into a cell may be less than the level necessary for detection of the CD21 antigen by current antibody staining methods. Second, it is possible that NK cells express the EBV receptor transiently during development, as recently described for human thymocytes, allowing infection of the malignant cell at an earlier stage of development. An increased number of NK cell progenitors available for potential EBV infection may result during the induction of NK cells during the response to infection. Finally, EBV may gain entry by alternative means, possibly an Fc receptor-dependent mechanism (novel NK cells express a high density of FcγRIII) or other unidentified receptors present on NK cells or their progenitors. Natural infection of T cells or their CD21+ progenitors might therefore account for the few examples of EBV-associated T-cell disease, including T-cell lymphoma, lethal midline granuloma, and Castleman's disease, and Kawasaki-like disease.

The demonstration of clonal EBV episomal DNA within the malignant LGLs implies that the proliferation of this monoclonal cellular population occurred after EBV infection and raises the possibility of an etiological role for EBV in the leukemic transformation. It is quite possible that the EBV transforms LGLs in a manner akin to B lymphocytes, although the molecular mechanism whereby EBV transforms the LGLs must for the moment remain speculative. Neither ENBA-2, which is required for the immortalization of B lymphocytes, nor LMP-1, which is oncogenic in rodent cell lines, was detected in the leukemic LGLs. This pattern of EBV gene expression is similar to that in EBV-associated Burkitt’s lymphoma and the majority of cases of nasopharyngeal carcinoma. A postulate akin to that suggested for Burkitt’s lymphoma might argue that a viral infection, possibly EBV itself, led to an expanded LGL precursor pool allowing entry of EBV into some LGLs, one of which subsequently acquired another unidentified oncogenic molecular change. The presence of EBV gene products, eg, LMP-1, EBNA-2, might have ensured survival of that cell, allowing the leukemic LGL clone to emerge. Subsequent immunologic recognition of antigen-positive cells may have driven selection of the EBV-positive LGL clone to the EBNA-2, LMP-1-negative phenotype described here. Alternatively, the EBV-infected LGL cells may have expressed only EBNA-1 and the EBV-encoded small, nonpolyadenylated RNAs (EBERs), gene products that may alone confer some oncogenic properties, from the beginning.

This case of EBV-associated aggressive LGL proliferation in combination with the report of EBV in other LGL proliferations suggests that useful information may be obtained by investigating LGL proliferations more widely for EBV infection. It should also prompt further experiments to investigate whether EBV can infect NK cells during their differentiation.

**ACKNOWLEDGMENT**

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