Abstract

C.B-17 scid mice were reconstituted by intraperitoneal injection of human tonsil cells or PBL from EBV-seronegative donors. Subsequent injection of EBV resulted in the rapid development (within 19–33 d) of aggressive, fatal, lymphoproliferative disorders of human B cell origin. Autopsies revealed solid tumors in the abdomen, and occasionally in the liver, thymus, or spleen. Histopathologic analysis showed that the tumors were high-grade immunoblastic lymphomas and FACS analyses of tumor cells indicated that they were of human B-lymphoid origin. The tumor cells grew in vitro and induced new tumors on injection into severe combined immunodeficient (SCID) mice. Karyotypic analysis and Southern blots for c-myc or bcl-2 rearrangements revealed no chromosomal abnormalities and translocations. Southern blot analysis also showed that the cells possessed EBV DNA sequences. Although these tumors undoubtedly reflect infection of the transferred B cells with EBV in vivo, intraperitoneal transfer of short-term lymphoid cell lines transformed in vitro with EBV resulted in ascites production without evidence of tumor formation. (J. Clin. Invest. 1990. 85:1333–1337.)

Introduction

Epstein-Barr virus (EBV), a human herpesvirus, is strongly associated with two human malignancies, Burkitt’s lymphoma and nasopharyngeal carcinoma. EBV has also been implicated in the B cell lymphomas and lymphoproliferative disorders that occur in immunosuppressed recipients of organ allografts (1–3), and in AIDS patients with disease-associated immunosuppression (4, 5). While the EBV genome is generally present in the proliferating cells in these conditions, there is limited information on its role in pathogenesis, since the natural host range is limited to man. Although new world monkeys, particularly cotton-top tamarins, develop tumors and lymphoproliferative disease 14–21 d after EBV injection (6–8), these animals are endangered and not generally available. In this paper we describe a novel model for EBV-induced human lymphoproliferative disease. We report that EBV infection of mice with severe combined immunodeficiency (SCID) (9) reconstituted by intraperitoneal injection of PBL from EBV-seronegative donors (or tonsil cells) is followed 19–33 d later by the development of aggressive fatal lymphoproliferative disorders of human B cell origin. The tumors contain EBV and are histologically similar to human EBV-positive posttransplant lymphomas. Unlike the recently reported spontaneous development of human B cell lymphoblastic lymphomas in SCID mice 2–4 mo after reconstitution with PBL from EBV-seropositive but not seronegative donors (10), the contributions of EBV, the target B cell and T cell immunity can be rapidly and independently assessed in this new model. In common with another recent description of SCID/hu chimeras (10), we found no evidence of graft-versus-host disease; the reasons for this unexpected xenospecific anergy are currently unknown.

Methods

Epstein-Barr virus. Supernatants of PMA-treated B95-8 cell cultures were used directly or concentrated by centrifugation at 60,000 × g for 90 min. All virus preparations were frozen in liquid N2 before use.

Human lymphocytes. PBL from venous blood or tonsil cells from therapeutic tonsillectomies were obtained by Ficoll-Hypaque separation. The EBV immune status of blood donors was assessed with a commercial assay for serum antibody against EBV capsid antigen (Electro-Nucleonics, Inc., Columbia, MD).

Mice. C.B-17 scid (SCID) mice (9), aged 8–12 w, were from the Research Institute of Scripps Clinic breeding colony, which is free of mouse hepatitis virus. SCID mice were reconstituted by intraperitoneal injection of at least 5 × 106 human PBL or tonsil cells; such mice are hereafter referred to as SCID/hu chimeras.

Recovery of tumor cells; in vitro culture. Cells were recovered from solid tumors by treatment of minced tissue with collagenase and trypsin, after a previously described method (11). Tumor cells were cultured in RPMI 1640 supplemented with antibiotics, glutamine, and 10% FCS (Hyclone, Logan, UT).

Lymphoid cell lines (LCL). EBV-transformed LCL were prepared by infection of PBL with B95-8 virus in the presence of Cyclosporin A (Sandoz, East Hanover, NJ), as previously described (12).

Antibodies. The following monoclonal antibodies were used for flow cytometric (FACS) analysis of tumor cells: anti-CD20 (Coulter, Hialeah, FL); RR1/1 (anti-LFA-3) and TS2/9.1 (anti-ICAM-1), kindly provided by Dr. T. A. Springer (Harvard Medical School); OKT3 (anti-CD3), HB5 (anti-CD21), 3D9 (anti-CD35), and M1/42 (against a species-specific common determinant on mouse MHC class I molecules) from the American Type Culture Collection (Rockville, MD).

1. Abbreviations used in this paper: LCL, lymphoblastoid cell line; SCID, severe combined immune deficient (mice).
FITC-(Fab')2 goat anti-human lgG and IgM (H & L) were from Accurate Corp. (New York). Goat anti-human kappa or lambda light chain was from Organon Teknika, West Chester, PA. A FACS IV analyzer (Becton-Dickinson Co., Rutherford, NJ) was used for flow cytometry.

**Histopathology.** Tissue samples were fixed in B-5 formalin (6% HgCl2, 12.5% CH3COONa, 4% formaldehyde), and sections stained (hematoxylin/eosin) or subjected to immunohistochemical analyses with antibodies specific for human B cells.

**Southern blot assays.** Tumor cell DNA was analyzed by Southern blots as described (13), using specific 32P-labeled DNA probes. 20 μg of tumor cell genomic DNA was digested with Eco RI or Msp I, electrophoresed in 0.7% or 1.7% agarose gels, respectively, and transferred by the alkaline blot method to nylon membranes, which were hybridized with the DNA probes. EBV DNA was detected with an Msp I fragment of the B95-8 Bam HI K region (coding for EBNA-1), from a pBR322-EBV recombinant library (14). Probes for c-myc and bcl-2 oncogenes were from Oncor, Gaithersburg, MD.

**Results**

**EBV infection of SCID/hu chimeras.** PBL from four EBV-seronegative donors (designated J. R., D. P., N. H., and T. C.) and tonsil cells from one donor of EBV status (age 4 yr) were transferred intraperitoneally to SCID mice. The mice were injected 7–16 d later with a 40-fold concentrate of B95-8 supernatant (usually 150 μl i.p. and/or 150 μl i.v.), except for the recipients of 4-yr-old tonsil cells, which received 1 ml of B95-8 supernatant (0.8 ml i.p., 0.2 ml i.v.). These experiments are summarized in Table I.

All 10 SCID/hu chimeric recipients of intraperitoneally injected EBV (with or without concomitant intravenous EBV injection) developed clinical signs of disease 19–33 d later. Autopsies revealed that all EBV-infected chimeras had abdominal solid tumors (3–15 mm diam), often closely associated with the liver; additional tumors were found in the thymus or spleen of some mice. No signs of disease were observed up to day 67 after intravenous injection of EBV (Table I), indicating that the intraperitoneal route of EBV injection is primarily responsible for lymphoproliferative disease. Uninfected SCID/hu chimeras have shown no clinical signs of disease up to 109 d postreconstitution with human cells.

**Histopathologic characterization of tumors.** Examination of stained sections indicated that tumors were high-grade large B cell immunoblastic lymphomas with conspicuous plasma-cytoid cytologic atypia and well-defined areas of necrosis (Fig. 1 A). They were thus similar to human EBV-positive post-transplant lymphoproliferative disorders. Immunohistochemical analyses indicated that the tumor cells were of human B cell origin (not shown).

**FACS analysis.** In every experiment, viable cells were recovered from the tumors and cell lines established. Phenotypic analysis by flow cytometry confirmed that the cells were of B lymphoid origin; cells were negative for CD20, CD21, and for human Ig expression (not shown). Where examined, tumor cells expressed low levels of both kappa and lambda Ig light chains, indicating that the tumor cells were oligoclonal or polyclonal. High levels of ICAM-1 and LFA-3 expression, typical of EBV-transformed LCL (15), were also observed (not shown). Tumor cells were negative for human CD3 and mouse class I MHC molecules. FACS analysis also excluded the possibility of inadvertent transfer of live B95-8 cells to the SCID mice: tumor cells (and LCL) were positive for CD35 and ICAM-1, whereas MAb for these markers failed to stain B95-8 cells.

**Genetic analysis.** Preliminary karyotype analysis of cells from one of the tumors (by Dr. O. W. Jones, University of California, San Diego) showed them to be normal diploid cells without chromosomal abnormalities. The number of metaphase spreads analyzed would have detected abnormalities in 25% or greater of the cells. Supporting this observation, Southern blot assay on D. P. and J. R. SCID/hu chimeric tumor cells revealed no evidence of c-myc or bcl-2 rearrangements (not shown). Southern blots also showed that tumor cells possessed B95-8 EBV Bam HI K (EBNA-1 coding region) DNA sequences.

**Adaptive transfer of the tumors.** Cells were recovered from a SCID/hu (donor J. R. PBL) tumor, cultured and expanded.

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<tr>
<th>Table I. EBV Infection of SCID/hu Chimeras</th>
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<td><strong>Cells transferred (5 x 10⁶, i.p.)</strong></td>
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<td><strong>Donor D.P. PBL</strong></td>
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<td><strong>Adoptive transfer of SCID/hu tumor cells</strong></td>
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through four weekly passages in vitro and transferred intra-
peritoneally to three SCID mice (5 × 10⁶ cells/mouse). All
three mice became severely ill 13–15 d posttransfer, and au-
topsy demonstrated that all three had abdominal tumors. Via-
able cells were recovered, and shown by FACS analysis to be
human B lymphoid cells. It was thus possible to reproduce
disease and tumor induction by cell transfer, even after several
cell passages in vitro.

Transfer of EBV-transformed LCL to SCID mice. Since the
tumors described above were undoubtedly a direct result of
EBV infection of B cells in vivo, it was considered important
to determine whether LCL derived by in vitro infection and
transformation with EBV could also produce tumors in SCID
mice. LCL established from three individuals were used in
transfer experiments, a synopsis of which is provided in Table
II. Two of the LCLs, designated J. R. and D. P., respectively,
were autologous to PBL used for SCID/hu chimeras. Although
intraperitoneal EBV injection of the J. R. or D. P. SCID/hu
PBL chimeras resulted in solid tumor formation, as described
above, intraperitoneal transfer of 5 × 10⁶ J. R. or D. P. LCL to
SCID mice induced ascites production 14–21 d later. Autop-
sies did not reveal any other gross pathology and there was no
evidence of tumor formation. Only 1–2 ml of ascites fluid
could be aspirated from the peritoneal cavity, but > 1 × 10⁸
cells were consistently recovered. FACS analysis indicated that
the majority (> 90%) of these cells were of human B lymphoid
origin. A notable feature of the ascites cells was that expression
of the adhesion molecules ICAM-1 and LFA-3 was markedly
depressed relative to the parent LCL. Ascites cell cultures in
vitro showed little evidence of the clumping that is character-
istic of LCL; this was presumably a reflection of ICAM-1 and
LFA-3 downregulation.

J. R. and D. P. LCL were newly established lines, and had
been in culture for about 1 mo. In contrast, the third LCL
(designated R. S.), transferred to SCID mice was a long term
cell line, having been continuously passed for almost a year
since its inception. RS LCL (3 × 10⁶) were transferred intra-
peritoneally to each of three mice. Remarkably, no disease
was seen in these mice until day 70 posttransfer, whereupon
the first mouse became ill and was sacrificed. Autopsy re-
vealed tumor masses in the abdomen, liver, and thymus.
Similar gross pathology was observed in the second and third
mice (days 81–86). Flow cytometry confirmed that the tumor
cells were human B lymphoid cells. Histopathologic analysis
indicated that the tumors were large cell immunoblastic
nonplasmacytoid lymphomas, with less differentiation than
the tumors seen after EBV infection of SCID/hu chimeras
(Fig. 1 B).

Discussion
We describe a new model for EBV-induced aggressive fatal
human lymphoproliferative disease in SCID mice reconsti-

Figure 1. Histologic characteristics of SCID/hu tumors. (A) Abdominal tumor appearing after EBV injection into SCID mice reconstituted with
human tonsil cells. Plasmacytoid immunoblasts and a well-defined eosinophilic zone of necrosis (lower field) are evident. (B) Less differentiated
nonplasmacytoid tumor following transfer of long-term RS. LCL into SCID mice. Hematoxylin/eosin stain, ×40.
tuated with human leukocytes. Resultant tumors were of human B cell origin, and possessed EBV DNA sequences; histopathologically the tumors were large B cell immunoblastic lymphomas with prominent plasmacytoid differentiation and extensive well-defined areas of necrosis. These features are shared by human posttransplant lymphoproliferative disorders (16-18). Preliminary analysis of Ig light chain expression indicated that the SCID/hu tumors were oligoclonal or polyclonal; organ transplant associated lymphoproliferative disorders have been found to be monoclonal, multilocal or polyclonal by analyses of cellular Ig expression (17), Ig gene rearrangements (18), or EBV terminal repeats (3). It has been suggested that monoclonal tumors may develop from a polyclonal hyperplastic background (3, 17).

Organ transplant-associated lymphoproliferative disorders develop in immunosuppressed individuals with impaired EBV-specific T cell immunity (1). The SCID/hu chimeras clearly lack EBV-specific T cell immunity as they are reconstituted with EBV seronegative cells. Although uncontrolled proliferation of EBV infected B cells would occur under these conditions, such proliferation alone would not produce the aggressive solid tumors characteristic of the posttransplant disorders in man and the EBV-infected SCID/hu chimeras described here. The additional factor(s) responsible for tumor development remain to be identified; neither the postransplant disorders nor the SCID/hu tumors possess the c-myc or bcl-2 chromosomal translocations characteristic of Burkitt's lymphoma and human follicular lymphomas, respectively.

Although the tumors in the SCID/hu chimeras undoubtedly reflect EBV infection of B cells in vivo, in vitro EBV-transformed short-term LCL produced asites but not tumors. The explanation for the markedly different biological consequences of in vivo and in vitro EBV infection is not known.

A long-term LCL line behaved differently, however, and produced tumors 70-86 d after transfer. Long-term LCL have previously been reported to be tumorigenic in nu/nu mice (19).

In summary, we have developed a novel animal model for EBV-induced human lymphoproliferative disorders and lymphomas. The model most closely resembles the EBV-related lymphoproliferative disorders that represent a frequent complication in immunosuppressed individuals receiving organ transplants, but it may also be a model for certain other EBV-associated lymphomas in man. Since the roles of EBV, the target B cell, and T cell immunity can be independently and rapidly assessed in this model system, further studies will yield insights into the role of each in the development of human lymphoproliferative disorders.

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References


