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Research Article

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Epstein-Barr Virus Induces Aggressive Lymphoproliferative Disorders of Human B Cell Origin in SCID/hu Chimeric Mice

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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.) Epstein-Barr virus • B cells • lymphoma • SCID mice

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)¹ (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 5×10^7 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).

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1. *Abbreviations used in this paper:* LCL, lymphoblastoid cell line; SCID, severe combined immune deficient (mice).

FITC-F(ab)₂ goat anti-human IgG 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 unknown 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 plasmacytoid 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 positive 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 assays on D. P. and J. R. SCID/hu chimera 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.

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

Table I. EBV Infection of SCID/hu Chimeras

Cells transferred (5 × 10 ⁷ , i.p.)	No. of mice	EBV Infection		Gross pathology
		Time	Route	
Donor D.P. PBL	2	Day 10	i.p., i.v.	2/2 clinically ill 28–31 d after EBV infection. Tumors in abdomen, spleen, thymus (2/2).
Donor J.R. PBL	2	Day 7	i.p., i.v.	2/2 clinically ill 19–20 d after EBV infection. Tumors in abdomen (2/2).
Donor N.H. PBL	3	Day 7	i.p., i.v.	3/3 clinically ill (one found dead) 20–21 d after EBV infection. Tumors in abdomen (3/3), thymus (1/3).
Donor T.C. PBL	3	Day 16	i.p.	3/3 clinically ill 24–30 d after EBV infection. Tumors in abdomen/liver, thymus (3/3).
Donor T.C. PBL	3	Day 16	i.v.	1/3 no clinical signs up to day 67. 2/3 ill days 43–45; 2 sacrificed; no tumors.
Tonsil cells (4-yr-old donor)	4	Day 16	i.p., i.v.	4/4 clinically ill (one found dead) 25–33 d after EBV infection. Tumors in abdomen (4/4).
Adoptive transfer of SCID/hu tumor cells				
Donor J.R. SCID/hu tumor cells 4th pass in vitro	3	—	—	3/3 clinically ill 13–15 d posttransfer. Tumors in abdomen (3/3).

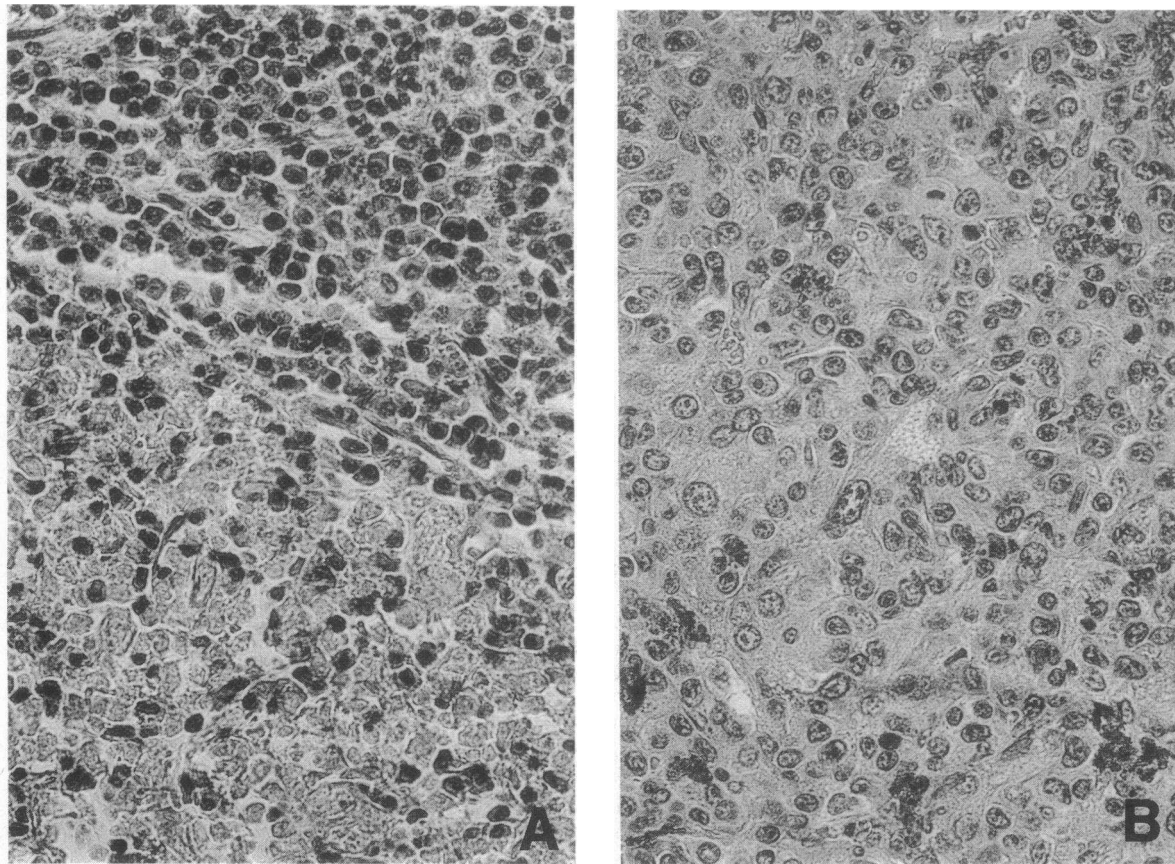


Figure 1. Histologic characteristics of SCID/hu tumors. (A) Abdominal tumor appearing after EBV injection into SCID mice reconstituted with human tonsil cells. Plasmacytoid immunoblasts and a well-defined eosinophilic zone of necrosis (*lower field*) are evident. (B) Less differentiated nonplasmacytoid tumor following transfer of long-term RS. LCL into SCID mice. Hematoxylin/eosin stain, $\times 40$.

through four weekly passages *in vitro* and transferred intraperitoneally to three SCID mice (5×10^6 cells/mouse). All three mice became severely ill 13–15 d posttransfer, and autopsy demonstrated that all three had abdominal tumors. Viable 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 5×10^6 J. R. or D. P. LCL to SCID mice induced ascites production 14–21 d later. Autopsies 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 $> 1 \times 10^8$ 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 characteristic 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 passaged for almost a year since its inception. RS LCL (3×10^6) were transferred intraperitoneally 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 revealed 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-

Table II. Transfer of EBV-transformed LCL to SCID Mice

Cells transferred	No. of mice	Time of clinical disease	Gross pathology
5 × 10 ⁶ J.R. LCL	3	Days 19–21	No tumors Ascites (2/3) 1/3 healthy up to day 69
5 × 10 ⁶ D.P. LCL	3	Days 14–17	No tumors Ascites (2/3) one found dead day 17
3 × 10 ⁶ R.S. LCL	3	Days 70–86	Tumors in abdomen, liver, thymus (3/3) No ascites* (2/3)

* A few cells (4 × 10⁶) recovered 1/3.

tuted 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, multiclonal 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-injected SCID/hu chimeras described here. The additional factor(s) responsible for tumor development remain to be identified; neither the posttransplant 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 ascites 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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