Epstein-Barr Virus (EBV)-associated Lymphoproliferative Disease in the *SCID* Mouse Model: Implications for the Pathogenesis of EBV-positive Lymphomas in Man

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Summary

When human peripheral blood lymphocytes (PBLs) from Epstein-Barr virus (EBV)-seropositive donors are injected intraperitoneally into SCID mice, EBV$^+$ B cell tumors develop within weeks. A preliminary report (Mosier, D. E., R. J. Gulizia, S. M. Baird, D. D. Richman, D. B. Wilson, R. I. Fox, and T. J. Kipps. 1989. *Blood.* 74(Suppl. 1):52a) has suggested that such tumors resemble the EBV-positive malignancy, Burkitt's lymphoma. The present work shows that generally the human (hu) PBL-SCID tumors are distinct from Burkitt's lymphoma and instead resemble lymphoblastoid cell lines (LCLs) generated by EBV-infection of normal B cells in vitro in terms of: (a) their cell surface phenotype, with expression of B cell activation antigens and adhesion molecules, (b) normal karyotype, and (c) viral phenotype, with expression of all the transformation-associated EBV latent proteins and, in a minority of cells, productive cycle antigens. Indeed, in vitro–transformed LCLs also grow when inoculated into SCID mice, the frequency of tumor outgrowth correlating with the in vitro growth phenotype of the LCL which is itself determined by the identity of the transforming virus (i.e., type 1 or type 2 EBV). Histologically the PBL-derived hu-SCID tumors resemble the EBV$^+$ large cell lymphomas that develop in immunosuppressed patients and, like the human tumors, often present at multiple sites as individual monoclonal or oligoclonal foci. The remarkable efficiency of tumor development in the hu-SCID model suggests that lymphomagenesis involves direct outgrowth of EBV-transformed B cells without requirement for secondary genetic changes, and that selection on the basis of cell growth rate alone is sufficient to explain the monoclonal/oligoclonal nature of tumor foci. EBV$^+$ large cell lymphoma of the immunosuppressed may arise in a similar way.

The C.B-17 *scid/scid* mutant strain of mouse (hereafter referred to as the SCID mouse) exhibits a severe combined immunodeficiency characterized by the absence of mature B and T lymphocytes (1); indeed, the mutation is thought to affect a component of the recombinase enzyme system involved in Ig and TCR gene rearrangements (2–4). The unexpected finding that mice carrying the SCID mutation could be grafted successfully with human (hu) haematopoietic cells has opened the way to the use of the hu-SCID chimeric mouse as a model for studying human immune-function in vivo (5–7). In addition, one of the original papers describing such engraftment showed that SCID mice reconstituted with high numbers of (hu-PBL-SCID mice) often developed lymphomas of B cell origin if the donor lymphocytes were from individuals with serological evidence of infection with EBV; these tumors were shown to carry EBV DNA but were otherwise poorly characterized (5). It was immediately apparent from

these studies that SCID mice could provide a potentially interesting in vivo model of human B lymphomagenesis involving an important viral pathogen.

EBV is a human herpesvirus that in vitro has potent growth transforming activity for resting B lymphocytes but that in vivo is carried as an asymptomatic and persistent infection by >90% of adults worldwide. The virus is, however, consistently associated with two types of B cell malignancies: (a) large cell lymphomas which arise in immunocompromised individuals such as post-transplant patients (8, 9), and (b) the high incidence, or "endemic," form of Burkitt's lymphoma (BL)[1] which is geographically restricted to equatorial regions of Africa and Papua New Guinea where malaria is holoen-

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[1] *Abbreviations used in this paper:* BL, Burkitt's lymphoma; LCL, lymphoblastoid cell lines; NGS, normal goat serum; NRS, normal rabbit serum; VCA, viral capsid antigen complex.

demic (10). Large cell lymphomas express a cell surface phenotype similar to that of lymphoblastoid cell lines (LCLs) generated by infection of normal resting B cells in vitro with EBV (11, 12); this is characterized by high cell-surface expression of various B cell–associated "activation" antigens such as CD23 and CD39, and of the adhesion molecules LFA-1 (CD11a/18), LFA-3 (CD58), and ICAM-1 (CD54). In contrast, BL cells express none of these LCL-associated antigens, with the possible exception of LFA-1; instead they display other surface markers (e.g., CD10 and CD77) consistent with their probable germinal center origin (13). Furthermore, large cell lymphomas appear to express the same spectrum of EBV latent gene products as are constitutively expressed in all in vitro–transformed LCLs, i.e., six nuclear antigens (EBNA 1, 2, 3a, 3b, 3c, and -LP) and two membrane-associated proteins (LMP-1 and LMP-2), while BL cells express detectable levels of EBNA 1 only, with downregulation of the other EBV latent genes (14, 15).

These differences between the two forms of EBV$^+$ lymphoma in man reflect their different pathogenesis. Thus, large cell lymphoma appears to be primarily a virus-driven lymphoproliferation, in which the outgrowth of EBV-infected transformed B cells is possible because the host control mechanisms that normally prevent such outgrowth, in particular the EBV-specific cytotoxic T cell response (16), are impaired. The fact that such tumors can sometimes present as monoclonal foci (9, 17, 18) raises the possibility of a second genetic event, in addition to EBV-induced growth transformation, occurring before the emergence of a malignant clone. There is as yet no firm evidence for such an event, and the tumors do not show any consistent chromosome abnormalities. On the other hand, endemic BL clearly has a multistep pathogenesis involving: (a) EBV infection of a particular target cell pool, (b) chronic malarial infection of the host, which causes temporary immunosuppression (19) and chronic stimulation of germinal center activity (20–22), and (c) a chromosome translocation involving chromosome 8 and either chromosome 14, 2, or 22, which results in deregulated expression of the c-myc protooncogene (23, 24), thereby giving the affected cell an additional proliferative advantage.

Given these differences between EBV-associated large-cell lymphomas and BL, it was of interest to characterize more fully the EBV$^+$ tumors that arise spontaneously in SCID-hu chimeric mice, particularly in the light of a preliminary report suggesting that such tumors are essentially BL-like (25). That report, and the fact that AIDS patients can in some instances develop classic EBV$^+$ BL (26), raised the possibility that EBV-infected BL progenitor cells are more frequent in asymptomatic virus carriers than had been previously thought.

Materials and Methods

Isolation of Human Lymphocytes and Generation of Cell Lines In Vitro. PBLs were isolated from heparinized peripheral blood by isopycnic centrifugation on Ficoll-Hypaque. Spontaneous LCLs carrying the donors own resident EBV strain were established by culturing PBLs from EBV-seropositive individuals in the presence of Cyclosporin A to avert any T cell–mediated regression of outgrowth

(27). Other LCLs were generated by deliberate in vitro infection of PBL cells from EBV-seronegative individuals with different isolates of EBV (28).

Generation of Human B Cell Tumors in SCID Mice. In the main set of experiments, between 35 and 50 × 10^6 PBLs, isolated from healthy donors of known EBV serological status, were washed with PBS and injected intraperitoneally into SCID mice. In other experiments, 5 × 10^6 cells from established LCLs were injected intraperitoneally. If animals became sick they were killed and autopsied. Tumors and other tissues of interest were dissected into two or more pieces which were either: (a) fixed in 10% formaldehyde/saline for immunohistologic analysis, (b) used for preparing viable cell suspensions for cell surface phenotype analysis or for karyotyping, or (c) snap-frozen in liquid nitrogen for later preparation of cryostat sections for immunofluorescence studies and for preparing DNA and/or protein samples for Southern and Western blots, respectively.

Histopathology and Immunohistochemistry. Tumors fixed in 10% formal saline were processed to 2-μm paraffin wax–embedded sections for staining with hematoxylin-eosin, or 3-μm sections for immunohistochemical staining. A routine avidin-biotin complex (ABC) immunostaining schedule was used with the primary murine mAbs UCHL1 (CD45RO antibody; DAKO Ltd., High Wycombe, UK) for the detection of T cells, and L26 (Bionuclear Services, Reading, UK) for the detection of B cells. In addition, a standard alkaline phosphatase anti–alkaline phosphatase (APAAP) method was used with the mAb BU38 (The Binding Site Ltd., Birmingham, UK) for the demonstration of CD23.

Cell Surface Immunofluorescence of Tumor Cell Suspensions. For the preparation of viable cell suspensions, lumps of tumor weighing ~200 mg were cut into several small pieces with a scalpel, resuspended in RPMI 1640 tissue-culture medium (no serum) containing 0.05% collagenase (No. C9891; Sigma Chemical Co.), and agitated for 45 min at 37°C. The cell suspension was separated away from the remaining undigested tissue and washed twice in medium containing 10% FCS. Typically, at least 10–20 × 10^6 viable cells were recovered. These cells were labeled with mAbs specific for cell surface antigens, followed by FITC-conjugated goat anti–mouse IgG antibodies as described (29). The stained cells were analyzed on a FACS 440 (Becton Dickinson & Co., Mountain View, CA). The panel of mAbs used included 55 (CD10, CALLA), AC2 (CD39), RR/1 (CD54, ICAM-1), and TS2/9 (CD58, LFA-3); these mAbs were used in an earlier study to characterize the cell surface phenotype of BL cells (29).

Southern Blot Analysis for Ig Gene Rearrangements. DNA was prepared from snap-frozen biopsy material (30) and 10 μg DNA was digested with BglII restriction enzyme (Bethesda Research Laboratories, Gaithersburg, MD). The digested DNA was electrophoresed on a 0.8% agarose slab gel and transferred to Hi Bond N$^+$ filters (Amersham Corp., Arlington Heights, IL) by capillary blotting in 0.4 M NaOH. Filters were prehybridized at 65°C for 4 h in 2× SSPE (360 mM NaCl, 20 mM Na$_2$HPO$_4$, 2 mM EDTA), 5% dextran sulphate, 1% SDS, 0.5% dried skim milk. Random primed ^{32}P-labeled C$_k$ probe and 100 μg/ml denatured salmon sperm DNA were added and the hybridization was continued for 20 h. The C$_k$ probe was a 0.75-kb HindIII/EcoRI insert of pRH10, a gift from T.H. Rabbitts, MRC, Cambridge, UK. Filters were washed in 2× SSC (150 mM NaCl, 15 mM sodium citrate) for 15 min at 20°C, for a further 20 min at 60°C in 1× SSC, with 1% SDS, and briefly in 0.5× SSC with 1% SDS at 60°C. Hybridized probe was detected by autoradiography of the filters for up to 10 d using Kodak X-Omat S film.

Immunofluorescence for Human κ and λ Ig Light Chains on Frozen Tissue Sections. Acetone-fixed cryostat sections of 5 μm thickness

were rehydrated with PBS containing 20% normal goat serum (PBS/NGS), and then incubated for 1 h at 37°C with a mixture of TRITC-conjugated goat antibodies to κ Ig light chains and FITC-conjugated goat antibodies to λ Ig light chains (Nordic Immunological Laboratories Ltd.), diluted 1:10 in PBS/NGS. After washing the slides in PBS, they were mounted in DABCO-based anti-fading mountant (31) and visualized by fluorescence microscopy.

Immunofluorescence for EBV Antigens on Frozen Tissue Sections. The following mAbs were used for the detection of virally encoded proteins: CS.1-4 (anti-LMP) (32), PE2 (anti-EBNA 2) (11), JF186 (anti-EBNA-LP) (33), BZ.1 (anti-BZLF1) (Young, L. S., R. Lau, M. Rowe, et al., manuscript in preparation), and V3 (anti-VCA) (34). Methanol-fixed cryostat sections of 5 μm thickness were rehydrated with PBS containing 20% normal rabbit serum (PBS/NRS) and incubated with specific mAbs for 2 h at 37°C. Bound mAb was detected by incubation with FITC-conjugated goat anti–mouse IgG (No. F0257; Sigma Chemical Co.) for 1.5 h at 37°C, followed by washing in PBS, and then for a further 1 h at 37°C with FITC-conjugated rabbit anti–goat IgG (No. F2016; Sigma Chemical Co.). The mAbs were diluted either 1:1 (hybridoma culture supernate) or 1:100 (ascitic fluids) in PBS/NRS, while the FITC conjugates were diluted 1:50 in PBS/NRS containing 10% EBV-seronegative human serum. The slides were finally washed in PBS, mounted in DABCO-based anti-fading mountant (31), and visualized by fluorescence microscopy.

Western Blot Analysis for EBV Gene Expression. Frozen pieces of tumor biopsies were prepared for Western blotting by first homogenizing in ice-cold Ripa buffer (0.5% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 10 mM Tris buffer pH 8.0) at 5% weight/volume, and then diluting with an equal volume of 2× gel sample buffer (4% SDS, 10% β-mercaptoethanol, 0.02% bromophenol blue dye, 0.1 M Tris buffer, pH 6.8). The samples were then sonicated and boiled for 2 min and 40-μl aliquots were loaded per track for electrophoresis. Control samples were prepared from 10^6 washed cells from cultured cell lines by sonicating and boiling in 20 μl of 1× gel sample buffer. SDS-PAGE on 7.5% acrylamide Laemmli gels, followed by blotting onto nitrocellulose filters, probing with specific antibodies and detection with ^{125}I–protein A, was performed as described elsewhere (35). In addition to the mAbs to EBV latent infection proteins described above, a polyspecific human serum, PB, from a chronic infectious mononucleosis patient was used to detect EBNA 2, EBNA 3a, EBNA 3b, and EBNA 3c. Monospecific antibodies to EBNA 1 were affinity-purified from pooled human sera by affinity chromatography on a synthetic peptide, p107, which contains glycine/alanine repeat sequences of EBNA 1 (36).

Immunofluorescence Test for Anti-VCA Antibodies. Human IgG antibodies to the EBV-encoded viral capsid antigen complex (VCA) were detected by a standard indirect immunofluorescence procedure (37) using acetone-fixed smears of P3HR-1 (producer line) and Raji cells (non producer line) as target antigens.

Results

In the main set of experiments, aliquots of 35–50 × 10^6 PBLs isolated from one of eight EBV-seropositive donors were injected intraperitoneally into SCID mice; some 17 animals were reconstituted in this way. PBLs from all eight donors gave rise to tumors; these arose in 14 (82%) of the mice within a mean time of 83 ± 25 d (range 63–120 d). Multiple tumors were often found, commonly presenting at the base of the liver, in association with the gut, and in the thymus. Exten-

sive necrosis of the liver and an enlarged spleen were observed in all 14 tumor-bearing mice, as well as in one animal which became sick at 5 wk with no obvious tumors. The remaining two animals, together with three mice injected with PBLs from an EBV-seronegative donor, remained healthy after 19–23 wk and showed no evidence of tumors at autopsy.

In a parallel set of experiments, LCLs first generated in vitro, either by spontaneous transformation of cultured PBLs from seropositive donors or by experimental infection of PBLs from seronegative donors with EBV, were tested for their ability to grow in SCID mice. All of these LCLs, with the exception of the long-established X50-7 line, were from early-passage cultures. Many of the LCL-engrafted animals developed tumors (see later for details) and these were characterized along with the PBL-derived tumors.

Histopathology of Tumors. Sections stained with hematoxylin-eosin revealed that PBL-derived tumors in SCID mice possessed identical morphology to tumors arising in SCID mice injected with EBV-transformed cells from established LCLs (Fig. 1, *a* and *b*). The neoplasms were composed of a polymorphic mixture of lymphoplasmacytoid, lymphocytic, immunoblastic, and plasmacytic cells. These appearances are typical of polymorphic lymphoplasmacytoid non-Hodgkins lymphoma (Kiel classification). The morphology was identical for all tumors studied and is consistent with the histological description of EBV$^+$ large cell lymphomas arising in immunosuppressed patients (8, 9, 11, 12, 18).

Immunohistochemistry of Tumors. In the PBL-derived tumors, there were scant, scattered T cells of human origin which stained with the mAbs UCHL1 (Fig. 1 *c*) and MT1 (data not shown); these T cells were absent from the LCL-derived tumors (Fig. 1 *d*). All specimens, whether of PBL or LCL origin, labeled strongly with the pan-B mAbs L26 (Fig. 1, *e* and *f*) and MB2 (data not shown), and with the anti-CD23 mAb BU38 (Fig. 1, *g* and *h*).

Cell Surface Phenotype of Dispersed Tumor Cells. Viable cell suspensions from collagenase-dispersed tumors were analyzed for cell surface phenotype by indirect immunofluorescence staining with mAbs. The four mAbs used were selected from a panel previously used to discriminate between the phenotype of BL cells and LCL cells (15), and Fig. 2 shows FACS profiles obtained with these mAbs. The tumor cells from hu-PBL-SCID mice were CD10$^-$, but positive for the CD39 activation antigen and the CD54 (ICAM-1) and CD58 (LFA-3) adhesion molecules (Fig. 2 *A*). In comparison with cells taken from a culture of a spontaneous LCL from the same donor, the fluorescence intensity of staining for CD39, CD54, and CD58 was noticeably lower on the tumor cells from hu-PBL-SCID mice. However, a similar reduction of fluorescence intensity was often observed in tumors derived from LCLs injected into SCID mice. Thus, Fig. 2 *B* shows that PBL-derived tumors and LCL-derived tumors display a similar cell surface phenotype, and that this phenotype is distinct from that of BL cells which are strongly CD10$^+$ and essentially negative for CD39, CD54, and CD58.

Clonality of the Tumors. Cryostat sections of 13 tumors from PBL-reconstituted SCID mice were stained for κ Ig light chains using rhodamine-conjugated polyclonal antibodies and
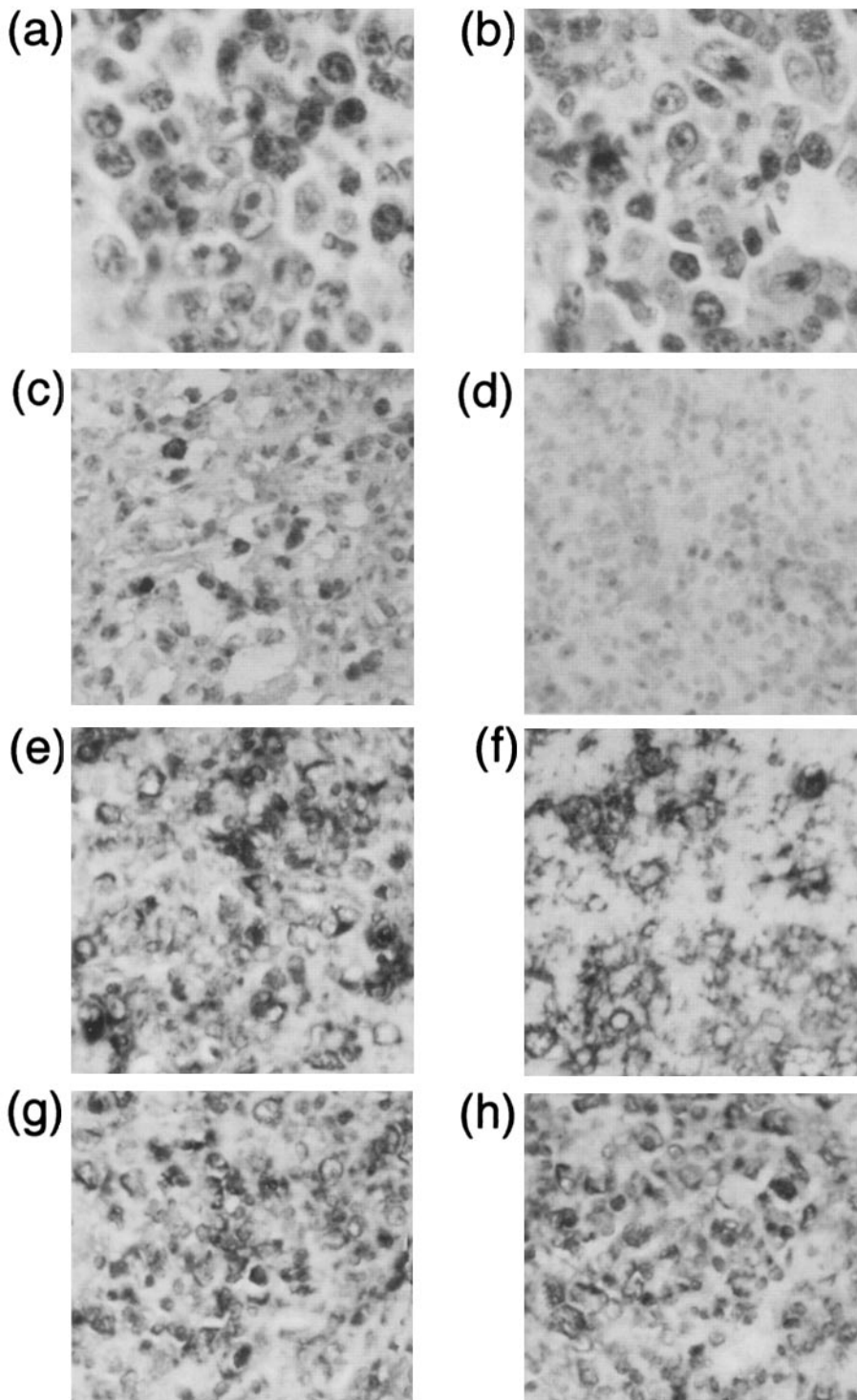
Figure 1. Histopathological and immunophenotypic characterization of formalin-fixed paraffin-embedded sections from tumors arising in a hu-PBL-SCID mouse (*a, c, e, g*) and in a hu-LCL-SCID mouse (*b, d, f, h*), respectively. Sections were stained with hematoxylin/eosin (*a, b*) or were immunostained with a pan-T cell mAb, UCHL1 (*c, d*), with a pan-B cell mAb, L26 (*e, f*), or with an anti-CD23 mAb, BU38 (*g, h*).

for λ light chains using FITC-conjugated polyclonal antibodies. Five of the tumors appeared to be monoclonal by this type of analysis, as illustrated by the tumor 2 derived from donor PLo (Fig. 3 *A*), while three of the tumors were clearly either polyclonal or oligoclonal, as illustrated by the tumor derived from donor MRe (Fig. 3 *B*). Five other tumors stained predominantly for one light chain. Analysis of DNA rear-

rangements in the Ig gene locus, which is a more definitive marker for B lymphoid clonality (38), confirmed that some tumors were indeed monoclonal. Thus, Fig. 3 *B* shows results obtained with three independent tumors (all of which were monoclonal by immunophenotyping) taken from one mouse 15 wk after engraftment with 35×10^6 PBLs from donor PLo. In this example, the DNA was digested with BglII and

A

CD10   CD39   CD54   CD58

SCID
PBL-Tumor

LCL

B

Akata-BL

SCID
PBL-Tumor

SCID
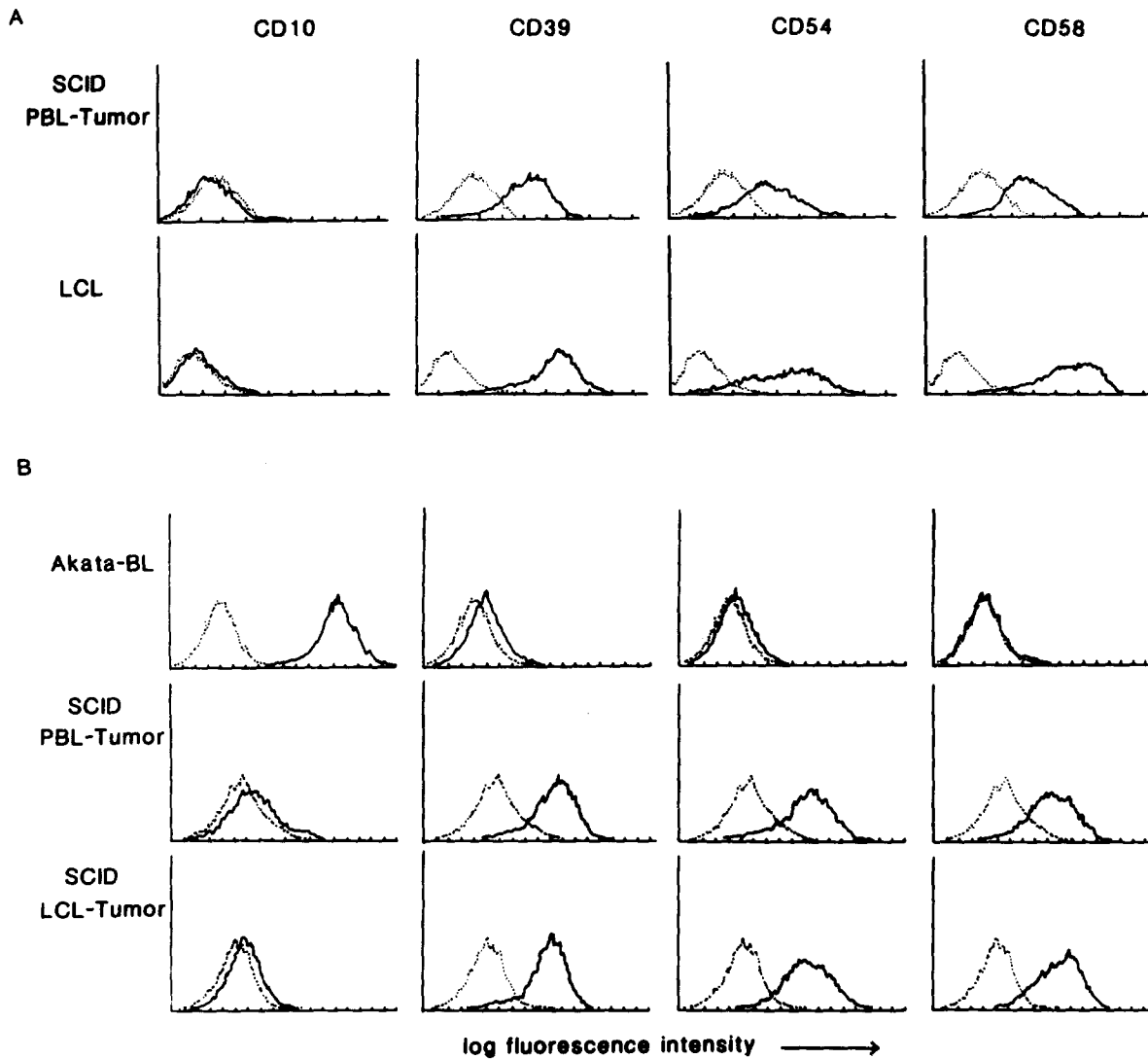LCL-Tumor

log fluorescence intensity ———→

Figure 2. FACS profiles of collagenase-dispersed viable cells from SCID-hu tumors, stained for CD10 (CALLA), CD39, CD54 (ICAM-1), and CD58 (LFA-3). Fluorescence intensity is expressed on an arbitrary logarithmic scale. (*A*) SCID mouse tumor cells derived from PBLs of donor HGa, compared with collagenase-treated cells of a cultured LCL established in vitro by spontaneous outgrowth PBLs of the same donor. (*B*) Cells from the Akata-BL line, which has retained a BL biopsy-like phenotype, compared with SCID mouse tumors derived either from PBLs of donor ADa or from an in vitro transformed LCL.

hybridized with a C_k probe. DNA prepared from Hela cells was used as a control unrearranged DNA sample, while spleen cells from the hu-PBL-SCID mouse were largely composed of murine cells and therefore DNA prepared from this tissue did not hybridize with the probe. The pattern of hybridization of the probe with DNA from the three PLo hu-PBL-SCID tumors indicated that each tumor contained a single predominant clone of B cells, and that individual tumors from the same animal could be clonally distinct. A cell line (PLo Tumor 3c) that was established in culture from the PLo tumor 3 showed the same pattern of Ig gene rearrangement as the original biopsy. The results obtained with the MRe tumor (Fig. 3 *B*) were consistent with this tumor being polyclonal or oligoclonal.

EBV Latent Gene Expression in PBL-derived SCID Tumors. In the first instance, EBV latent gene expression was investigated by Western blot analysis of tumors using a polyspecific human serum, PB, with reactivity against several of the EBNAs. Fig. 4 *A* shows representative results of such an analysis obtained with two independent tumors derived from PBLs from donor ADa; a spontaneous LCL, established from PBLs of the same donor in vitro, was included for comparison. The PB serum detected EBNA 2 and EBNA 3a,b,c proteins in both PBL-derived tumors, and the size of these proteins corresponded exactly to those expressed in the ADa-spLCL, thus confirming that these tumors carried the expected ADa EBV isolate. Note that two reference LCLs included in the same immunoblot carry different EBV isolates and express characteristically
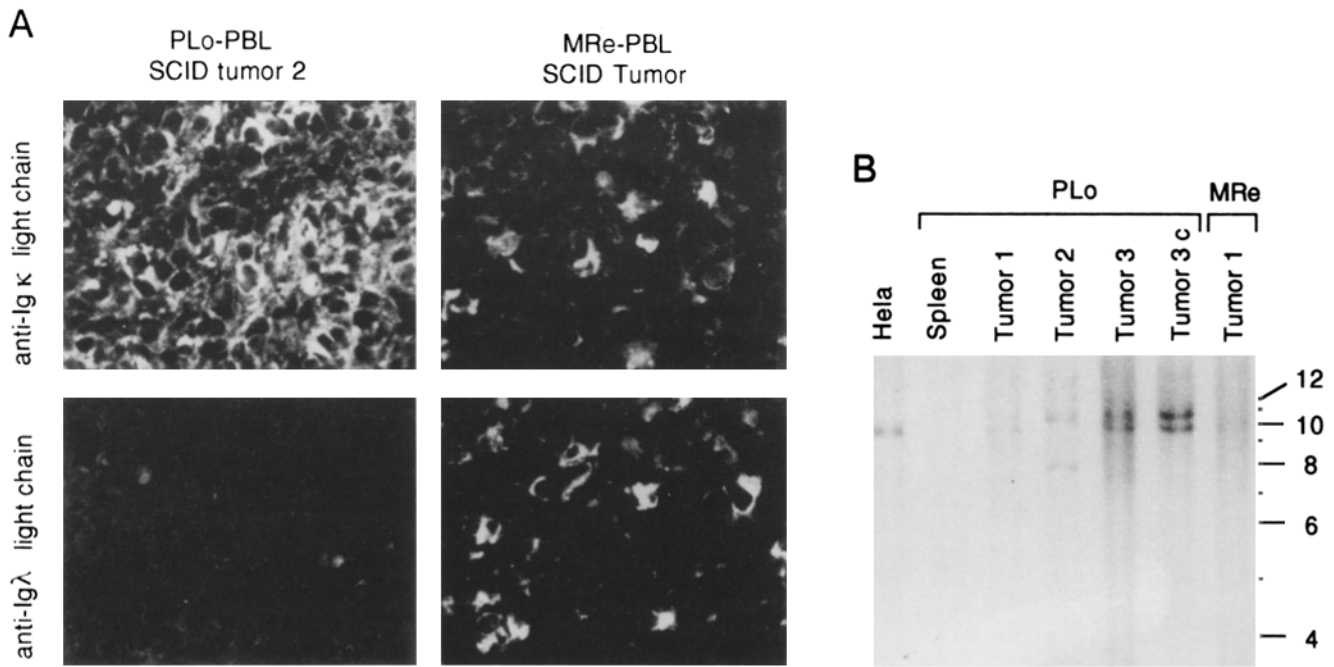
Figure 3. (*A*) Immunophenotyping of SCID mouse tumors derived from PBLs of donors PLo and MRe. Cryostat sections were analyzed by dichromatic fluorescence using polyclonal antibodies to Ig K (TRITC conjugate) and Ig λ (FITC conjugate). The two tumors correspond to PLo Tumor 2 and the MRe Tumor in the Southern blot illustrated in Figure B. (*B*) Southern blot of BglII-digested DNA, probed with a $C_κ$ probe, demonstrating Ig gene rearrangements in PBL-derived hu-SCID tumors. The molecular size markers (kbp) are indicated to the right of the blot. DNA from the Hela cell line, which served as a germline Ig gene control, was compared with DNA prepared from: the spleen (which lacked tumor cells) and three independent tumors from one mouse inoculated with PBLs from donor PLo, and one tumor arising in a second mouse inoculated with PBLs from donor MRe. In addition, an early-passage cultured line established in vitro from the PLo- tumor 3 was analyzed (PLo tumor 3c).

different sized EBNA proteins, while the EBNA 2 and EBNA 3a,b,c proteins were not detected in the EBV⁺ cell line Akata-BL, reflecting its BL origin.

Expression of the other EBV latent proteins in PBL-derived tumors was examined further using monospecific or monoclonal reagents since these avoid the problems of interpretation such as are illustrated in Fig. 4 *A* in the 25–60-kD region of the blot where PB serum may be detecting either EBNA-LP of the latent cycle, or the EA(D) complex of the lytic cycle, or degradation products of EBNAs 2 and 3. Thus, Fig. 4 *B* shows a similar blot probed with affinity-purified human antibodies to EBNA 1, and demonstrates that the same 2 representative ADa PBL-tumors expressed EBNA 1, as did the reference LCL and BL cell lines. Other blots probed with mAbs to EBNA2 (data not shown), to EBNA-LP (Fig. 4 *C*), and to LMP (Fig. 4 *D*) demonstrated the presence of these three latent viral proteins in the PBL tumors and in the LCL lines, but not in the Akata-BL line. Note that the JF186 anti-EBNA-LP mAb (Fig. 4 *C*) detects a ladder of different sized proteins that is characteristic for a given line but which may differ even between lines carrying the same EBV isolate; this reflects the variable multiplicity of repeat exons in different EBNA-LP mRNAs (33, 39). In addition, the JF186 mAb is known to react only with the EBNA-LP encoded by a minority of EBV isolates (e.g., the ADa EBV isolate, but not the isolate carried by the reference LCL 1

in Fig. 4 *C*): of the eight donors used as a source of PBLs for these SCID mouse studies, three donors carried EBV isolates encoding an EBNA-LP that was recognized by JF186, and the hu-PBL-SCID tumors derived from these donors were thus shown to express EBNA-LP. The expression of EBNA-LP in PBL-derived tumors from the other donors was verified using affinity-purified human antibodies (15).

The expression of EBV latent viral proteins in the majority of cells in all SCID-hu tumors tested was readily confirmed by immunofluorescence staining of cryostat tissue sections with mAbs to LMP, EBNA 2, and EBNA-LP (Fig. 5). PBL-derived tumors from each of the eight EBV-seropositive donors studied were shown, by one or both of the above techniques, to express the latent viral proteins that are constitutively expressed in LCLs but not in BL cells.

EBV Lytic Cycle Gene Expression in PBL-derived SCID Tumors. Western blots of PBL-derived tumors were probed with a human serum, EE, which has high titer antibodies to the immediate-early antigen BZLF1, and to the early antigen complex EA(D). As illustrated by the blot in Fig. 6, all of the tumors were found to express lytic cycle antigens to a greater or lesser extent. These data were confirmed by immunfluorescence staining with mAbs to the BZLF1 protein and to the viral capsid antigen VCA, which revealed a distinct subpopulation of antigen-positive cells distributed unevenly throughout the tumors (Fig. 7). Staining of cell
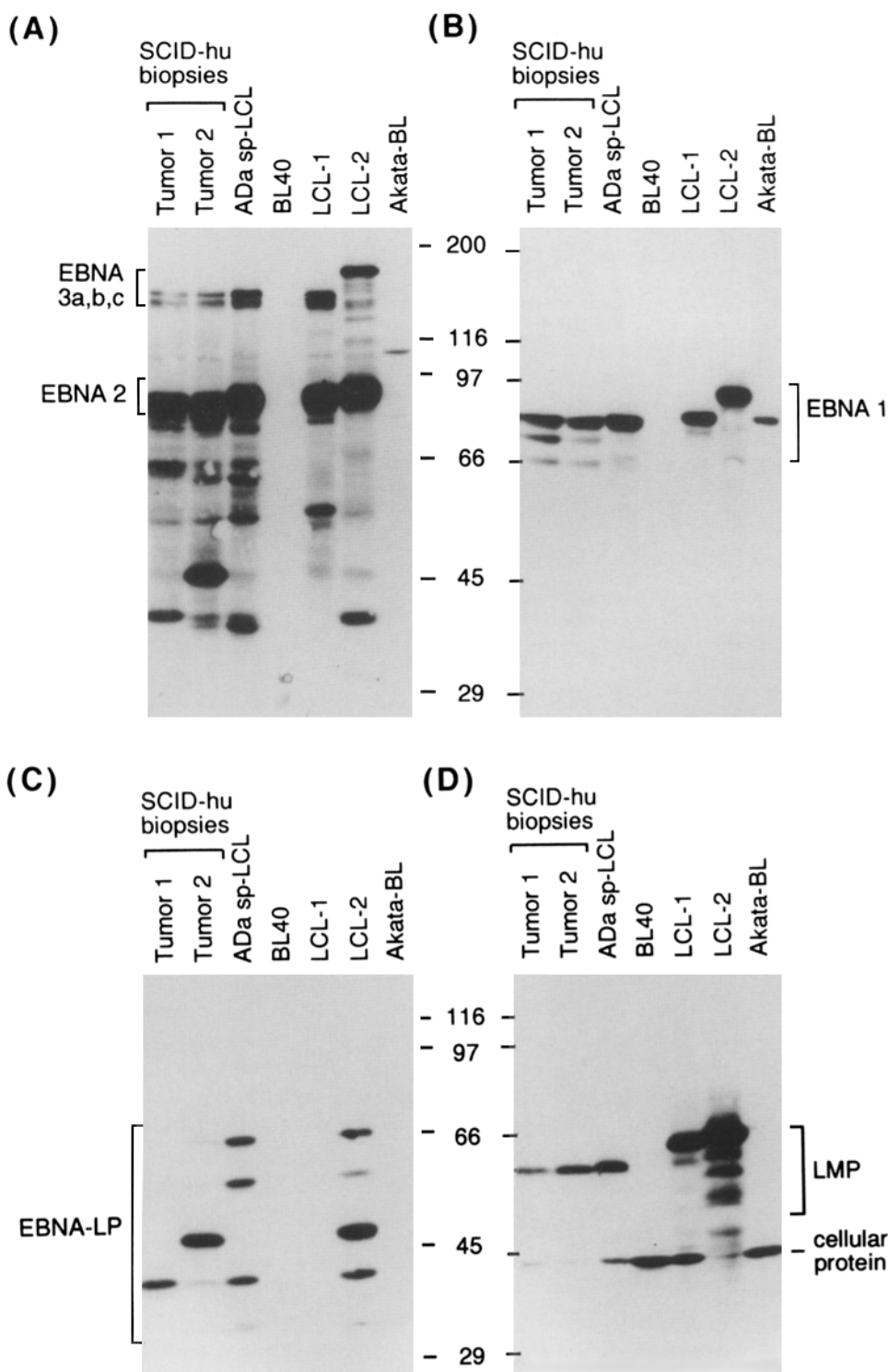
Figure 4. EBV latent proteins detected in PBL-derived SCID mouse tumors by Western blotting. Two independent tumors (tumor 1 and tumor 2) and an in vitro–established spontaneous LCL (spLCL) were all derived from PBLs of donor ADa. Other reference protein samples were prepared from: an EBV⁻ line (BL40), two LCLs transformed with different isolates of EBV (LCL-1 and LCL-2), and the Akata-BL line. The blots were probed with: (*A*) serum PB, a polyspecific human serum for the detection of EBNA 2, EBNA 3a, EBNA 3b, and EBNA 3c; (*B*) monospecific anti-p107 human antibodies to EBNA 1, isolated by affinity chromatography using a synthetic peptide corresponding to an EBNA 1 repeated a.a. sequence; (*C*) JF186, a mAb reactive with EBNA-LP; and (*D*) CS.1-4, a pool of mAbs reactive with LMP. The positions of molecular weight marker proteins (sizes given in kilodaltons) are indicated. Note that *A* shows a deliberate over-exposure of the blot in order to illustrate clearly the presence and size of the EBNA 3 proteins in the hu-SCID tumors from donor ADa; a shorter exposure also demonstrated that the EBNA 2 protein in the SCID tumors was the same size as the EBNA 2 in the ADa spontaneous LCL.

smears prepared from collagenase-dispersed cell suspensions showed that up to 5% of the cells within a tumor may be in the virus-productive cycle (data not shown). No background reactivity was observed with these mAbs in normal tissues lacking tumor cells.

Humoral Response to Late Viral Antigens in hu-PBL-SCID Mice. The virus-producer status of the PBL tumors in hu-PBL-SCID mice contrasts with the situation in man where large cell lymphomas arising in immunosuppressed patients are apparently negative for productive cycle antigens (11, 12). Because these patients retain serum antibody responses to late viral antigens, we tested the hu-PBL-SCID mice for similar responses. Human IgG anti-VCA antibodies were monitored in two mice, each injected with 40 × 10⁶ PBLs from donor
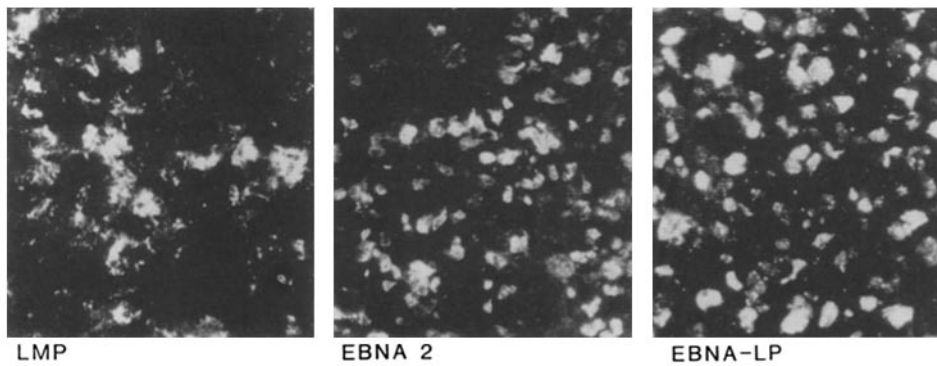
LMP   EBNA 2   EBNA-LP

Figure 5. EBV latent proteins detected in cryostat sections of a PBL-derived SCID mouse tumor (the same as the PLo tumor 2, shown in Fig. 3) by immunofluorescence staining with specific mAbs. LMP was detected with CS.1-4, EBNA 2 with PE2, and EBNA-LP with JF186.

MRe (Table 1). Low titers of anti-VCA antibodies were detected in both animals during the first 6 wk, but no antibodies were detected when tumors were identified at 10–11 wk. In total, the sera from nine animals injected with PBLs from five donors were tested at the time when killed, (range 10–23 wk), and anti-VCA antibodies were invariably undetectable.

Growth Properties of Cultured LCLs in SCID Mice. Taken together, the aforementioned data indicate that the tumors arising in SCID mice reconstituted with PBLs actually resemble in vitro–transformed LCL cells with respect to cellular phenotype and viral gene expression. This was in one sense surprising since it is well known that in vitro–transformed LCL cells do not grow when injected subcutaneously or intraperitoneally into nude mice (40). For this reason the present experiments were extended to test whether LCLs, like the PBL-derived tumors, were capable of growth in SCID mice. In an initial set of experiments tumors regularly developed in SCID mice injected intraperitoneally with 5×10^6 cells from each of three different LCLs. Two of these LCLs were established spontaneously in vitro from the PBLs of the EBV-seropositive donors, MRe and HGa, and were used in early passage (before passage 10), while the third LCL was a long-established line, X50-7, obtained by deliberate infection of cord blood cells in vitro with EBV. Each of the spontaneous LCLs was injected into a set of three SCID mice, while the X50-7 line was injected into two mice: all eight mice produced multiple solid tumors within 27 d which were histologically and phenotypically indistinguishable from PBL-derived tumors (see Figs. 1 and 2).

It is now clear that EBV isolates fall into two types (1 and 2), based on polymorphism of their EBNA2 and EBNA 3a, b, c genes (35, 41, 42); these viruses have slightly different biological properties in vitro such that type 1 EBV-transformed LCLs grow more quickly and to a higher saturation cell den-
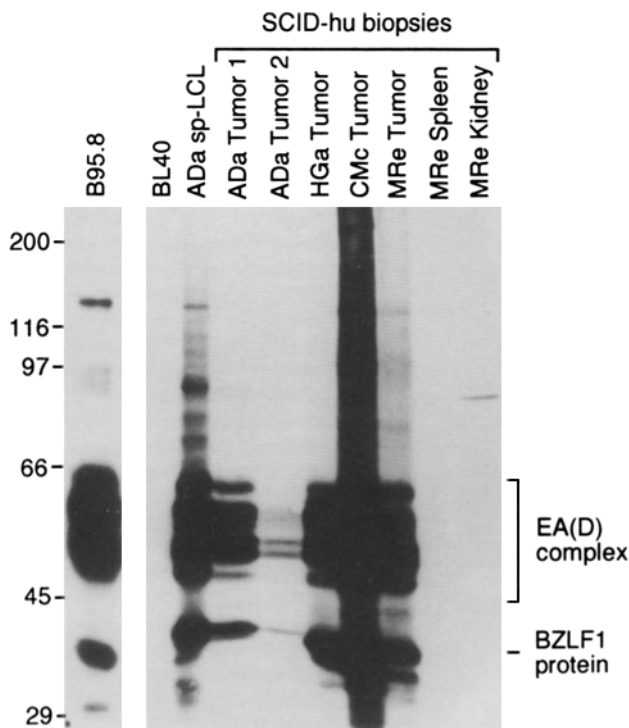

Figure 6. Western blot demonstrating the expression of EBV productive-cycle proteins in PBL-derived tumors from SCID mice. The blot was probed with the human serum EE, which has high titer antibodies to the BZLF1 immediate-early antigen and to components of the EA(D) early antigen complex. In this blot, five independent tumors derived from PBLs of four different donors (ADa, HGa, CMc, and MRe) are compared with extracts from spleen and kidney tissue that did not show any evidence of tumors. The B95.8 virus-producer cell line, which contains ~5% VCA+ cells, was run on the same gel for reference.

Table 1. *Anti-VCA Antibodies in SCID Mice Injected with PBLs from Donor MRe*

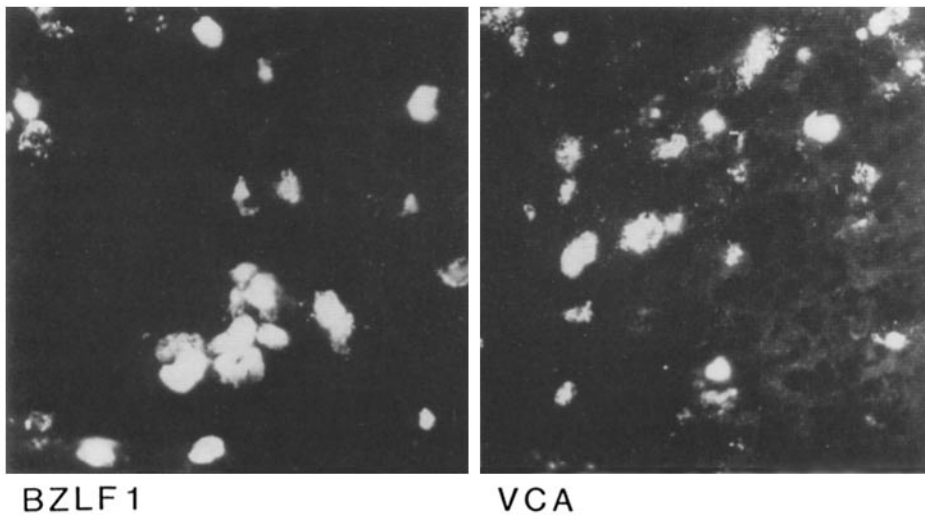| Serum sample		Reciprocal anti-VCA titer
Donor human serum,	day 0	960
SCID-hu mouse-1	3 wk	40
	6 wk	40
	10 wk	Negative
SCID-hu mouse-2	3 wk	20
	6 wk	30
	11 wk	Negative

BZLF1   VCA

Figure 7. EBV productive cycle antigens detected by immunofluorescence staining of cryostat sections of the same PBL-derived tumor as in Fig. 5. The BZLF1 protein was detected with the mAb BZ.1, and VCA was detected with the mAb V3.

sity than do type-2 transformants (28). Because all of the donors used as a source of PBLs for the main set of experiments were carrying the more common type 1 isolates of EBV, and because the three LCLs tested initially for growth in SCID mice were also type 1 transformants, we were interested to investigate the capacity of type 2 transformants to grow in this same environment. Therefore, eight different LCLs, all established at the same time from PBLs of a single EBV-seronegative donor by infection in vitro with different type 1 and type 2 isolates of EBV (28), were compared for their ability to induce tumors in SCID mice. The panel of LCLs consisted of four lines infected with different type 1 EBV isolates and four lines infected with different type 2 EBV isolates, all tested in early in vitro passage. Each LCL was injected into a set of three mice and of the animals injected with type 1 LCLs, 11 of 12 produced solid tumors within a mean time of 30.2 ± 7.1 d (range 20–43 d). In contrast, only 2 of 12 animals injected with type 2 LCLs developed tumors (at 38 and 52 d); the remaining 10 animals were healthy at the time when killed (109 d) and lacked any evidence of tumors at autopsy.

Discussion

The present analysis of human B cell lymphomas arising in SCID mice engrafted with PBLs from healthy EBV-seropositive individuals shows that these tumors closely resemble in vitro–transformed LCL cells and are distinct from BL cells by histological appearance (Fig. 1), cell surface phenotype (Fig. 2), and EBV gene-expression (Figs. 4 and 5). Furthermore, conventional karyotyping of metaphase spreads from three independent PBL-derived monoclonal tumors revealed normal diploidy and the absence of any chromosome translocations characteristic of BL (Stacey, M., and M. Rowe, unpublished observations). These results are in contrast to the suggestion made in a recent report (25), based on one PBL donor, that the tumors arising in SCID mice have some characteristics of BL: this particular donor may be an interesting

exception to the general rule. In our cell surface phenotype analysis, some PBL-derived tumors did show slightly reduced expression of LCL-associated markers such as CD39 and the adhesion molecules (Fig. 2 A), but a similar reduction was also observed in tumors generated in SCID mice after inoculation with in vitro transformed LCLs (Fig. 2 B). This minor shift in cell surface phenotype appears to be a consequence of the in vivo environment since lines established in culture from the tumors reverted to their usual high levels of antigen expression within a few in vitro passages (data not shown).

By the above criteria of histological appearance, cell surface phenotype, and EBV latent gene expression, the PBL-derived tumors in hu-PBL-SCID mice are similar to EBV+ large cell lymphomas that arise in immunosuppressed individuals. The one interesting difference between these two tumors is the unexpected finding that the lymphomas arising in the hu-PBL-SCID model contained a proportion of cells expressing EBV antigens of the virus-productive cycle (Figs. 6 and 7). Although Katz et al. (43) reported the presence of linear (i.e., replicating) EBV DNA in ∼40% of EBV-associated lymphomas in immunosuppressed patients, early and late viral proteins themselves have not been detected in such biopsies to date (11, 12). Similarly, EBV+ tumors induced in cotton-top tamarins by experimental infection with the virus in vivo do not express lytic cycle antigens, although cell lines derived from the tamarin tumors do become virus-productive in vitro (44). The PBL-derived tumors in SCID-hu mice therefore provide the first documented evidence of EBV productive cycle antigens being expressed in B cells in vivo. Note that in this animal model there are no detectable antibody responses to late viral antigens at the time of tumor development (Table 1). This distinguishes the hu-PBL-SCID model from both immunosuppressed humans and experimentally infected tamarins, and raises the possibility that antibody-mediated mechanisms may normally repress or eliminate cells entering the EBV productive cycle in vivo.

An important feature of the PBL-derived SCID tumors is their presentation as discrete foci at multiple anatomical sites,

each focus often being composed of a single unique B cell clone (Fig. 3); this feature is also shared by the large cell lymphomas in immunosuppressed patients (9, 17, 18). It has been argued that the monoclonality of many of the EBV⁺ lymphomas in immunosuppressed patients reflects the occurrence of a second genetic event, in addition to the essential EBV-driven proliferation, which results in clonal selection of the malignant cells. However, the rapidity with which EBV⁺ monoclonal tumors lacking chromosomal abnormalities can arise in the SCID mouse model, from an estimated input of ∼25–50 EBV-infected B cells in 50 × 10⁶ PBLs (27, 45), seems to us to argue against a specific second malignant event in the lymphomagenesis. It is well known that even during EBV-induced B cell growth transformation in vitro, where there is no obvious selection except on the basis of growth rate, the original polyclonal LCL can soon become dominated by one or a few clones with serial passage in culture (46). A similar selection on the basis of differential growth rates could well operate in vivo, both in human-SCID chimeric mice and in immunosuppressed patients. This is entirely in line with our observation that LCLs generated in vitro by infection with type-2 EBV isolates, and showing poorer in vitro growth than corresponding type 1 transformants (28), are much less efficient at forming tumors in SCID mice than are type 1 transformants. This may have important implications since type 2 isolates are now known to be present within Western communities, albeit at a much lower incidence than type 1 isolates (47). The inference is that immunosuppressed patients with type 2, rather than a type 1, EBV infection will be less susceptible to lymphoproliferative disease.

In many respects, EBV-associated lymphomagenesis in the hu-PBL-SCID model reflects the situation in vitro when lymphocytes from healthy EBV carriers give rise to LCLs by spontaneous outgrowth. This leads to some interesting predictions since it is known that such spontaneous outgrowth in vitro occurs by a two-step mechanism; EBV-infected B cells in the circulating pool enter the productive cycle on being placed in culture, and the virus thus released then infects and transforms other resting B cells coresident in the culture (48, 49). Lymphomagenesis in PBL-inoculated SCID mice may therefore be sensitive to early inhibition of virus replication by agents such as Acyclovir (50), or to passively transferred virus-neutralizing antibodies, both of which can reduce the incidence of spontaneous transformation in vitro. Spontaneous outgrowth of LCLs in vitro can also be prevented by an autologous EBV-specific cytotoxic T cell response, hence the need to include cyclosporin A to prevent memory T cell activation in vitro (27, 45). It would be anticipated that the lymphomas arising in hu-PBL-SCID mice would also be sensitive to such T cell control, and that the outgrowth of tumors in this model presumably reflects the poor survival of functional CD8⁺ cells in lymphoid organs (51, 52); certainly the minor population of infiltrating T cells observed both in the PBL-derived SCID tumors (Fig. 1) and in large cell lymphomas of immunosuppressed patients (12) cannot represent a functional EBV-specific cytotoxic T cell response.

In its wider aspect, the present work re-emphasizes the fact that in most adults the B cell pool is colonized by a virus with lymphogenic potential. This potential is only realized in special circumstances in vivo when immune impairment allows the outgrowth of EBV-transformed B cells with an LCL-like phenotype, as occurs in immunosuppressed patients and in SCID mice. There is no evidence from the present work that asymptomatic virus carriers harbor EBV-infected B cells that represent the immediate preneoplastic progenitors of BL. It is of course interesting that AIDS patients are not only at risk for the same EBV-driven large cell lymphomas as seen in other immunosuppressed patients but also for classical BL, at least a proportion of which are EBV genome-positive (26). We would argue that the increased incidence of BL is not a consequence of immunosuppression in these patients, but another feature of their condition, chronic hyperproliferation of germinal centers (53) affecting the very site from which the tumor is thought to arise (13).

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