Abstract

Epstein-Barr virus (EBV) is associated with B-cell malignancy in immunosuppressed humans and SCID mice receiving human peripheral blood leukocyte grafts (hu-PBL-SCID). We have further characterized the process of lymphoma development in hu-PBL-SCID mice. We report that EBV-seropositive donors differ markedly in the capacity of their PBL to give rise to immunoblastic lymphomas in SCID mice; some donors (high incidence) generated tumors rapidly in all hu-PBL-SCID mice, other donors (intermediate-low incidence) gave rise to sporadic tumors after a longer latent period (>10 weeks), and some donors failed to produce tumors. B-cell lymphomas arising from high incidence donors were monoclonal, whereas tumors from intermediate-low incidence donors were multiclonal. EBV replication was detected in all tumors. Tumors derived from intermediate-low incidence donors were monoclonal and had evidence of viral replication. All tumors, regardless of the donor, resembled EBV-transformed lymphoblastoid cell lines in surface phenotype but differed from lymphoblastoid cell lines by having less Epstein-Barr nuclear antigen 2 and CD23 expression. The variable patterns of lymphomagenesis seen among different EBV-seropositive donors may be explained by lower levels of specific immunity to EBV in high incidence donors, permitting activation of EBV replication and potential transformation of secondary B-cell targets. In addition, there may be differences in the transforming potential of EBV infecting different donors. The use of the hu-PBL-SCID model may help predict patients at high risk for posttransplant or acquired immunodeficiency syndrome-associated lymphomas.

Introduction

EBV, a human herpesvirus, is the causative agent of infectious mononucleosis (1) and has been implicated as a cofactor in two types of malignancies, nasopharyngeal carcinoma (2) and endemic Burkitt’s lymphoma in Africa (3). After primary infection, the virus usually persists in a latent state in B-lymphocytes and in an active replicative state in the oropharynx (4). Although latent infection is not associated with disease in immunocompetent individuals, EBV-induced B-cell lymphomas occur in a proportion of individuals on immunosuppressive therapy for organ transplants (5) and AIDS patients (6). These large cell immunoblastic lymphomas appear to express similar cell surface markers (CD23, LFA-1, ICAM-1) and EBV latent gene products (EBNA-1, EBNA-2, LMP-1) as those expressed by LCL transformed in vitro by EBV. EBV-associated posttransplantation lymphomas are thought to reflect the transforming capacity of viral latent genes in the absence of an EBV-specific T-cell response (7).

We have previously reported the successful engraftment of human PBL in immunodeficient scid/scid (SCID) mice as a model for studying human immune function in vivo (14). We observed the development of human B-cell lymphomas in mice receiving PBL from donors with serological evidence of EBV infection (14). Histopathologically, these tumors resembled large cell immunoblastic lymphomas arising in immunosuppressed patients, and EBV DNA was detected in all tumors (14, 15). These findings were confirmed by Rowe et al. (16), who showed that the tumors phenotypically resembled LCL and that some replication stage antigens were expressed, raising the possibility that EBV replication contributed to lymphomagenesis in hu-PBL-SCID mice.

Preliminary experiments in our laboratory led to the observation that PBL from some EBV-seropositive donors failed to generate tumors in SCID mice. This was of particular interest in the context of PTL, since the majority of immunosuppressed patients fail to develop EBV-associated PTL (17, 18). Accordingly, we initiated a study of ten healthy EBV-seropositive donors to determine if they exhibited variability in their capacity to generate B-cell lymphomas in hu-PBL-SCID mice. The results showed that substantial variability exists among EBV-seropositive donors in the ability of their PBL to generate tumors in SCID mice. Tumors derived from “high incidence” PBL donors who consistently and rapidly generated lymphomas showed evidence of EBV replication by Southern blot analysis using probes specific for the fused TR region of the EBV genome, which distinguishes between episomal (latent) and linear (replicating) forms of DNA (19-22). EBV replication was not detected in the most tumors derived from PBL donors who generated more slowly appearing lymphomas in only a fraction of hu-PBL-SCID mice. In addition, we found that expression of EBNA-2 and CD23 was substantially lower in hu-PBL-SCID lymphomas derived from all donors than in LCL.

Materials and Methods

Donors. Peripheral blood was collected from 10 healthy EBV-seropositive and 5 EBV-seronegative donors. EBV exposure was assessed by a standard indirect immunofluorescence procedure detecting antibodies to EBV viral capsid antigen (23).

Cells. Cell Lines, and Viruses. Human PBL were obtained from heparinized blood by isopycnic centrifugation through Histopaque (Sigma). PBL were washed twice and resuspended at 100 × 10⁶ cells/
ml in RPMI 1640 for injection. The B95-8 strain of EBV (24) was used to generate LCL by ly1 vitro infection of PBL in the presence of 1 µg/ml of cyclosporin A. LCL lines were established from all EBV-seropositive donors and one EBV-seronegative donor (donor 14 LCL = RG LCL). Daudi and Ramos are EBV-positive and EBV-negative Burkitt's lymphoma lines, respectively. LCL and tumor lines were grown in RPMI 1640 supplemented with 10% heat-inactivated FBS, 1 mm glutamine, 10 mm 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid and 50 µg/ml of gentamicin.

Mice. PBL (50 × 10⁶) or lesser cell numbers of in vitro transformed LCL were transfered i.p. into 8–10-week-old C.B.-17 scid/scid mice previously shown not to produce mouse immunoglobulin ("leaky" phenotype) as described (14). Mice were bled every 2 weeks and human immunoglobulin levels determined by ELISA. Mice with a continuous increase in human immunoglobulin levels (15) were closely watched for clinical signs of tumor formation. At this point mice were sacrificed and tumors were recovered. Tumors were processed for further analysis depending on their size. Typically, tumor sections were snap-frozen for future DNA, protein, or immunohistochemistry analysis, and cell suspensions were prepared for either FACS analysis or establishing tumor cultures. A limited number of tumors were fixed in 10% formalin-saline for further hematoxilin-eosin staining.

ELISA. To determine human IgG, IgM, IgA, κ or λ levels, 2-fold dilutions of hu-PBL-SCID sera or culture supernatants were incubated on polynvinyl chloride plates (Dynatech) previously coated with either goat anti-human IgG, IgM, IgA (heavy chain specific; Fisher Biotech), or goat anti-human total immunoglobulins (heavy and light (H + L) chain specific; Cappel) antibodies, respectively. Detection of specific antibodies was carried out as follows: goat-anti-human immunoglobulin-horseradish peroxidase conjugate (H + L chain specific; 1:200; Cappel) was used to detect the presence of the three different isotypes, while goat anti-human κ or λ-peroxidase conjugate (1:1000; Fisher-Biotech) was used for detection of light chains. Plates were developed with o-phenylenediamine and read at 492 nm. Human standards were always run in parallel.

FACS Analysis. Viable cell suspensions were prepared by dispersing ~200 mg of tumor tissue in RPMI 1640 containing 0.005% collagenase type VII (Sigma C-2399) by incubation at 37°C for 45 min. After being washed twice in RPMI 1640 supplemented with 10% heat-inactivated FBS, 2 × 10⁶ cells/antibody reagent were incubated in 10 µl of ice cold blocking buffer [17% rat anti-mouse Fcγ receptor (Pharmingen), 15% purified rat IgG1 and 17% normal mouse serum in flow buffer (Hanks' balanced salt solution containing 3% FBS, 0.1% sodium azide, and 10 mm 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)] for 20 min. Subsequently, an equal volume of flow buffer and 2 µl of the following directly fluorescein isothiocyanate-labeled mouse monoclonal antibodies were added: anti-human CD4, CD8, CD10 (CALLA), CD20, CD23, CD11a, CD54, HLA-DR, CD45 HLe-1 (Becton-Dickinson), and HLA-A,B,C (Sera-lab). Samples were also stained with a directly phycoery-thin-covalently coupled monoclonal rat anti-mouse H-2Kš antibody (Pharmingen) to detect the presence of mouse cells in the tumors. Tumors placed in culture and control cell lines were stained following the same procedure. Samples were analyzed using a FACScan (Becton Dickinson).

Western Blot. Protein samples were prepared by mincing frozen tumor sections in ice cold radiomunoprecipitation assay buffer (0.5% Nonidet P-40–0.5% sodium deoxycholate-0.1% SDS-150 mM NaCl-10 mm Tris buffer, pH 8.0) at 10% w/v. Subsequently, homogenates were mixed with an equal volume of 2× sample buffer (4% SDS-100 mM Tris buffer, pH 6.8–10% 2-mercaptoethanol-0.02% bromophenol blue dye) after which samples were sonicated and boiled for 3 min. Aliquots of 35 µl were loaded per lane for electrophoresis. Using the same procedure, control samples were prepared from cultured cell lines and the equivalent to 1.2 × 10⁶ cells was loaded per lane. SDS-polyacryla-mide gel electrophoresis was carried out on 7.5% acrylamide gels followed by blotting onto nitrocellulose filters. The following previously characterized monoclonal antibodies were used in the form of culture supernatants for detection of EBV-encoded latent proteins: S-12 (anti-LMP) (25) and PE-2 (anti-EBNA-2) (7). For detection of EBNA-1, serum from donor 2 was used. When tested against a B95-8-transformed LCL (RG), this serum showed reactivity against the same molecular weight protein as a previously characterized serum with EBNA-1 reactivity (26). A biotin-avidin system was used to detect the presence of specific antibodies. Briefly, blots were washed in PBS and incubated with either biotinylated goat anti-mouse IgG (1:500; Fisher Biotech) or goat anti-human immunoglobulins (1:500; Fisher Biotech) followed by a 30-min strepavidin-horseradish peroxidase (1:500; Fisher Biotech) incubation. After a washing, blots were developed with aminothiacyanate.

Southern Blots. DNA was prepared by phenol-chloroform extraction from frozen tumor sections as described previously (22). BamHI-digested DNA (10 mg) was electrophoresed on a 0.7% agarose slab gel and transferred to HyBond N+ filters (Amersham Corp.) by capillary blotting in 20 x SSC. Membranes were prehybridized at 42°C for 2 h in 5 x SSC, 5 x Denhardt's solution, 50 mm NaPO₄, 0.1% SDS, 250 µg/ml salmon sperm DNA, and 50% deionized formamide and hybridized under the same conditions for 18 h in the presence of a randomly primed 32P-labeled Jh, BamNJ, or BamW probe. The Jh probe corresponds to a 2.1-kilobase Sau3A fragment from the Jh germ line gene (27). Plasmids containing the EBV "BamW" (28) and "BamNJ" (29) regions were kindly provided by Dr. Jeffrey Cohen. The EBV genome contains a tandem array of 11 repetitive ("W") regions, each containing a unique BamHI restriction site (30). A BamHI digestion of EBV DNA will render one "C" (9.2-kilobase), one "Y" (1.9-kilobase) and 10 "W" (3.1-kilobase) fragments. The presence of 10 target sequences per genome makes hybridization with the BamW probe a sensitive approach for detecting EBV DNA. A detailed characterization of EBV DNA structure and BamNJ hybridization patterns is shown in Fig. 1. Filters were washed in 3 x SSC-1 mM containing 1% SDS for 15 min at 65°C and for a further 30 min in 1 x SSC-1 mM-1% SDS at 65°C. Hybridized probe was detected by autoradiography of the membranes using Kodak X-Omat S film.

RESULTS

Tumor Incidence with Different PBL Donors. We began these studies by comparing tumor incidence in 85 SCID mice reconstituted with PBL from 10 healthy EBV-seropositive donors and 4 healthy EBV-seronegative donors (experiment 1). In a second experiment carried out 10 months later, SCID mice derived from 3 of the EBV-seropositive donors and 1 EBV-seronegative donor used in the first experiment were reanalyzed for tumor formation. Similar results, which are summarized in Table 1, were obtained in both experiments. Tumors developed in hu-PBL-SCID mice derived from 8 of 10 EBV-seropositive donors, and no tumors were seen in any SCID mice generated using EBV-negative donors. In experiment 1, a total of 33 animals developed tumors during the 56-week observation period with a latent period ranging from 7 to 18 weeks. The frequency of hu-PBL-SCID mice developing tumors varied from 100% to 14% among different donors (Table 1). On the basis of these results, donors were grouped into 3 categories according to the frequency of SCID mice developing tumors following transfer of their PBL and the latent period until tumor appearance. High incidence donors (4 of 10 examined) gave rise to tumors in 100% of SCID mice with a relatively short (<10 week) latent period, intermediate-low incidence donors (also 4 of 10) gave rise to tumors in only a fraction of SCID mice with a longer latent period (>10 week), and no incidence donors (2 of 10) whose PBL failed to produce tumors in these experiments. These terms will be used to describe EBV-seropositive donors in subsequent discussion.

We analyzed IgG anti-viral capsid antigen titers and the number of B-cells (CD20+) or activated B-cells (CD20+, CD23+) in PBL among the different incidence donors and saw no correlation with ability to generate tumors (data not shown). High incidence donors tended to be older than no incidence donors.
HU-PBL-SCID LYMPHOMAS: DONOR HETEROGENEITY

A. CIRCULAR DNA

Bam NJ probe

N (+3.5 kb)

TR (-0.5 kb)

J (+8.0 kb)

CLONAL

NON-CLONAL

- 8.21 kb

- 4-6 kb.

B. LINEAR DNA

... 4-6 kb.

Fig. 1. EBV DNA structure and hybridization patterns [adapted from Baer et al. (30); not drawn to scale]. Inverted lollipops indicate BamHI restriction sites. The BamNJ probe corresponds to a 8.4-kilobase (kB) fragment subclone from λ NT3 (29). (A) Circular DNA. The DNA circularization process occurs in such a way that a variable number of TR, ~0.5 kilobase in size, are retained. At a low multiplicity of infection, a single episomal form will be present in each latently infected B-cell (20). Clonal tumor: If one type of episomal form is present throughout the tumor sample, a BmHI digest will give rise to a single fused DNA fragment usually >8 kilobases in size. Nonclonal tumor: If two or more circular forms that contain different numbers of TR are present, a family of bands will be detected. (B) Linear DNA. Linearization of EBV DNA occurs through cleavage of the episome in the region of the TR. If linear DNA is present, a ladder of 4-6-kilobase bands will be seen. -- -- --, on rare occasions, linear DNA can be detected outside this size range.

donors, but given the small numbers in each category, the difference was not statistically significant.

Tumors commonly presented as solitary or multifocal masses in the porta hepatis and less frequently in the mesentery, spleen, liver, and thymus. A continuous increase in human immunoglobulin levels in the serum of hu-PBL-SCID mice always preceded tumor development in mice derived from both high and intermediate-low incidence donors (Fig. 2), by contrast to mice not developing tumors where immunoglobulin levels were constant. Tumors derived from both high and intermediate-low incidence donors were classified as large cell immunoblastic lymphomas, in agreement with previous studies (15).

Surface Phenotype of hu-PBL-SCID Tumors and Tumor Lines. The cell surface phenotype of tumors arising in hu-PBL-SCID mice from both high and intermediate/low donors was similar, but not identical, to that of in vitro transformed LCL.

Table 1 Donor-related incidence and latency of lymphoma development in hu-PBL-SCID mice

<table>
<thead>
<tr>
<th>Donor</th>
<th>EBV status</th>
<th>Mice developing tumors/total</th>
<th>Time to tumor development Mean/ range (wk)</th>
<th>Donor classification</th>
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<tr>
<td></td>
<td></td>
<td>Experiment 1</td>
<td>Experiment 2</td>
<td>Experiment 1</td>
</tr>
<tr>
<td>1</td>
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<td>4/6</td>
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</tr>
<tr>
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<td>Positive</td>
<td>6/6</td>
<td>ND</td>
<td>8.7/8-10</td>
</tr>
<tr>
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<td>4/4</td>
<td>8.5/7-9</td>
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<tr>
<td>14</td>
<td>Negative</td>
<td>0/5</td>
<td>ND</td>
<td>NA</td>
</tr>
</tbody>
</table>

* NA, not applicable; ND, not done.
Fig. 3. FACS analysis of a representative hu-PBL-SCID primary (1°) tumor (donor 10), a tumor cell line derived from the same donor, and control LCL and Burkitt's lymphoma cell lines. A, staining of 1° tumor cells by anti-CD45 (---) or fluorescein isothiocyanate-labeled isotype control (-----). B, staining of 1° tumor cells by anti-CD20 (-----), anti-CD10 (-----) or fluorescein isothiocyanate-labeled isotype control (-----). C, staining of 1° tumor cells by anti-CD20 (-----), anti-CD10 (-----) or fluorescein isothiocyanate-labeled isotype control (-----). D, staining of mouse cells within the tumor by anti-mouse MHC class I (-----) or phycoerythrin-labeled isotype control (-----). E, staining of the same tumors cells shown in C after 6 weeks in culture by anti-CD23 (-----) or phycoerythrin-labeled isotype control (-----). F, staining of I° tumor cells by anti-CD8 (-----) or fluorescein isothiocyanate-labeled isotype control. G, staining of donor 3 LCL by anti-CD23 (-----) or phycoerythrin-labeled isotype control (-----). H, staining of Ramos cells with anti-CD10 (-----) or fluorescein isothiocyanate-labeled isotype control (-----). Between 1 and 2% human CD4 cells were detected in most of the tumors studied (data not shown).
relative levels of expression of various cell surface markers from tumors were compared to in vitro established LCL. The data are representative of high and intermediate-low incidence donors. All hu-PBL-tumors lines were analyzed 4-6 weeks after establishment in culture. ++, high level of expression; +, intermediate expression; +/−, low expression; −, no expression.

<table>
<thead>
<tr>
<th></th>
<th>CD20</th>
<th>CD23</th>
<th>CD11a</th>
<th>CD54</th>
<th>HLA-A,B,C</th>
<th>HLA-DR</th>
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<td>+/−</td>
<td>++</td>
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<td>+</td>
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<tr>
<td>LCL-tumors (in vivo)</td>
<td>+</td>
<td>+/−</td>
<td>+/−</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>Hu-PBL-tumors (in vitro)</td>
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<td>+/−</td>
<td>+/−</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>−</td>
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<tr>
<td>hu-PBL-tumors (in vivo)</td>
<td>+</td>
<td>+/−</td>
<td>+/−</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>−</td>
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</table>

Fig. 4. Mortality curves of SCID mice receiving in vitro transformed LCL or spontaneously occurring hu-PBL-SCID tumor cell lines. Groups of 4 animals received 10^5 or decreasing numbers of donor 3 LCL or tumor line 2 cells i.p. Both cell lines were maintained in culture for a period of 2 months prior to injection.

The presence of viral episomal (latent) and linear (replicating) DNA was investigated in hu-PBL-SCID tumors derived from the donors listed in Table 1 using Southern blot analysis of BamHI-digested DNA with the BamNJ probe. A total of 22 samples were analyzed (Figs. 5 and 6). With two exceptions (Fig. 6A), EBV DNA was detected by the BamNJ probe in all tumors. These two tumors were positive for EBV DNA using a more sensitive probe specific for the repetitive BamW region (data not shown). Multiple episomal DNA forms were found in all tumors derived from high incidence donors (donors 2, 3, 5, and 10) and some tumors derived from intermediate-low incidence donors (donors 1 and 4). Single episomal DNA patterns were observed among tumors from intermediate-low incidence donors (donors 1, 6, and 7). Linear DNA was readily detected in 11 of 13 tumors derived from high incidence donors and 2 of 7 intermediate-low incidence donors. Increased exposure times of the blots showed that linear DNA could be detected among all hu-PBL-SCID tumors derived from high incidence donors but only in 4 of 7 from intermediate-low incidence donors. Exceptions included tumors from donors 6 and 7 and tumor 1 from donor 1. To determine if linear DNA was present among LCL-derived tumors, samples obtained from the experiments described in Fig. 4 were analyzed. In contrast to primary hu-PBL-SCID tumors, no linear DNA forms were detected (Fig. 7). Infectious transforming virus was recovered from cell-free peritoneal lavages of several SCID mice harboring tumors derived from high incidence donors, confirming the viral replication inferred from the Southern blot analysis (data not shown).

Fig. 5. Southern Blot analysis with the BamNJ probe of tumors obtained from hu-PBL-SCID mice reconstituted with cells from donor 10. Negative controls include SCID mouse and human placental DNA. The Burkitt's lymphoma line Daudi represents an example of a monoclonal episomal DNA pattern and shows the absence of linear DNA. The less intense band in Daudi migrating as an ~23-kilobase (kb) fragment probably represents integrated viral DNA as previously described for Raji (20). Samples 3A and 3B are two independently processed fragments from the same tumor. As mentioned in Fig. 1, linear DNA is present outside the expected region in all three tumors.
EBV DNA were detected in all tumors, including tumor 6 (Fig. 8B), a finding consistent with the oligoclonal nature of B-cells in these tumors. To further resolve the issue of B-cell clonality, we analyzed immunoglobulin isotypes and κ/λ ratios in supernatants derived from short-term culture of hu-PBL-SCID tumors. Representative data are shown in Table 3. Most tumors from both high and intermediate-low incidence donors showed production of multiple isotypes and both κ and λ light chains, indicating their multiclonal origin. However, two tumors from donor 1 (intermediate-low incidence) produced immunoglobulin consistent with a monoclonal origin. These two tumors presented single episomal bands by BamNJ analysis further confirming their monoclonality (Fig. 6A). We therefore conclude that the majority of the spontaneous B-cell tumors appearing in hu-PBL-SCID mice are multiclonal in origin but that some tumors arising from intermediate-low incidence donors are monoclonal.

Expression of EBV Gene Products. The expression of EBV latent genes was investigated in hu-PBL-SCID tumors, tumor lines derived from spontaneous primary tumors (hu-PBL-SCID tumor lines), and LCL-derived tumors. EBNA-1 was expressed in all three cases (Fig. 9A; some data not shown). When hu-PBL-SCID tumor lines established from different donors were analyzed for EBNA-1 expression by Western blotting, molecules of different molecular weight were observed (different “EBNA types”) indicating differences among donor viral strains (32). No variability in EBNA-1 size was detected among tumor lines from the same donor (data not shown). Evaluation of EBNA-2 expression by Western blotting in tumor samples initially presented some difficulty. Fig. 9C shows that while EBNA-2 could be detected as a ~M, 90,000 protein in B95-8-transformed LCL or hu-PBL-SCID tumor lines propagated in culture (Fig. 9B), no EBNA-2 could be detected in freshly isolated hu-PBL-SCID and LCL tumors from the three groups of donors. To further evaluate EBNA-2 expression in these tumors, cryostat sections were analyzed by indirect immunofluorescence as described previously (7). Fewer than 10% of cells in spontaneous tumors and LCL-derived tumors expressed EBNA-2, as opposed to virtually 100% of in vitro maintained LCL (data not shown). LMP was detected in the majority of the cases as a ~M, 60,000 protein in both hu-PBL-SCID tumors and derived tumor lines (Fig. 9, B and D). Two hu-PBL-SCID lines established from donor 4 expressed low levels of LMP of increased electrophoretic mobility (Fig. 9B). It thus appears that spontaneous tumors arising in hu-PBL-SCID mice express low levels of (or have few cell expressing) EBNA-2, while levels of expression of LMP are more comparable to LCL. Our studies of tumor cell lines also indicate that all donors were infected by type A viruses (EBNA-2 ≈ M, 90,000), rejecting the possibility of type B EBV (EBNA-2 ≈ M, 72,000) infecting intermediate-low incidence donors (Fig. 9B).

DISCUSSION

The findings presented above extend our knowledge of EBV-associated B-cell lymphomas arising in hu-PBL-SCID mice in three areas: (a) we show that heterogeneity exists among healthy EBV-seropositive donors in their capacity to generate EBV-associated B-cell lymphomas in SCID mice; (b) donors whose PBL gave rise to tumors with high frequency invariably gave tumors with evidence of EBV replication, whereas tumors from intermediate-low incidence donors only infrequently
latent and replication stage antigens at the time of PBL transfer and the ability of that immune response, particularly T-cell immunity, to function in the context of the hu-PBL-SCID environment; and the level of activation of human B-cells in SCID mice. The fact that tumor incidence observed with a given donor was stable over time (see Table 1) indicates that transient activation of EBV in the donor is unlikely to play a role in the generation of these tumors. It is known that type A EBV has higher transforming potential than type B virus (34). Our results indicated that the donors studied were infected by type A viruses, which have higher transforming activity than type B viruses. However, there may be heterogeneity in the transforming activity among type A EBVs. Preliminary experiments suggest that tumor cell lines established from some intermediate-low incidence donors (e.g., donor 7) grow more slowly in secondary SCID recipients than tumor cell lines from high incidence donors. This observation contrasts with the finding that B95-8-derived LCL from all classes of donors, as well as tumor lines from high incidence donors, grew with similar kinetics in SCID mice. Variability in the number of latently infected B-cells has been reported among healthy EBV-seropositive individuals (4, 35, 36), and this might explain donor heterogeneity in giving rise to tumors in hu-PBL-SCID mice. Attempts to distinguish the number of latently infected B-cells between donors from the high and intermediate-low incidence groups have to date been unsuccessful; therefore we cannot resolve this issue.

The detection of EBV replication in the hu-PBL-SCID tumors was clearly correlated with the incidence of tumor generation among different EBV-seropositive donors. It appears from our data that although active replication of EBV was not required for tumor development (intermediate-low incidence donors, LCL tumors), it was found in all tumors arising from high incidence donors and its presence correlated with multiple episomal forms in hu-PBL-tumors (Figs. 5-7). These data also suggest that tumors arising from high incidence donors may involve more B-cell clones than those arising from intermediate-low incidence donors (Fig. 6), with the caveat that assessing tumor clonality by analysis of the number of viral episomal forms may potentially overestimate the number of B-cell clones in the presence of viral replication where superinfection can occur. Tumors arising from intermediate-low incidence donors and harboring only one EBV episomal form proved to be monoclonal by Jh rearrangement and analysis of secreted immunoglobulin (Table 3). These observations suggest two mechanisms for lymphomagenesis in hu-PBL-SCID mice: direct

| Isotype and light chain ELISA analysis of culture supernatants from hu-PBL-SCID tumors |
|---------------------------------|----------------|----------------|----------------|----------------|
| Donor 1                         | IgG | IgM | IgA | \(\kappa\) | \(\lambda\) |
| Tumor 1                         | +   | -   | -   | +             | -             |
| Tumor 2                         | +   | +   | -   | +             | +             |
| Tumor 3                         | +   | +   | -   | +             | -             |
| Tumor 4                         | -   | +   | +   | -             | -             |
| Donor 3                         |     |     |     |               |               |
| Tumor 2                         | +   | +   | -   | +             | +             |
| Tumor 3                         | +   | +   | -   | +             | +             |
| Tumor 6                         | +   | -   | -   | +             | +             |
| Tumor 7                         | +   | -   | -   | +             | -             |
| Donor 10                        |     |     |     |               |               |
| Tumor 1                         | +   | -   | -   | +             | +             |
| Tumor 2                         | +   | +   | -   | -             | -             |

Hu-PBL-tumor lines were allowed to grow to maximum density at which point culture supernatants were collected for immunoglobulin determination as described in "Materials and Methods." +, positive; - , negative.

showed EBV replication; and (c) the spontaneous B-cell lymphomas appearing in hu-PBL-SCID mice differed from EBV-transformed LCL in culture by having reduced EBNA-2 and CD23 expression.

Prior to this work, there was limited information on the ability of different donors to generate EBV-related lymphomas in SCID mice. In our original work (14) we had fortuitously employed three donors who would now be classified as high incidence (see Table 1). In the work of Rowe et al. (16), all eight donors studied gave rise to some tumors although the frequency of tumor formation in SCID recipients was reported only for one donor (82%). Okano et al. (33) observed tumors in 3 of 4 SCID mice given PBL from 2 different donors. The ten EBV-seropositive donors we studied could be classified into three groups based on the both the incidence of hu-PBL-SCID tumors and their latency period. Only 40% of EBV-seropositive donors examined fell into the high incidence category, while the remaining donors were divided between intermediate-low and no incidence categories. A total of 14 EBV-seronegative individuals (4 reported here) have been used to generate hu-PBL-SCID mice, and no tumors have been observed. What might explain the differences between donors with differing capacity to generate hu-PBL-SCID tumors? Several issues may bear on tumor incidence: the strain and transforming capacity of EBV infecting a given donor; the level of immunity to both
Fig. 8. Southern Blot analysis of tumors obtained from hu-PBL-SCID mice reconstituted with cells from donor 3. (A) JH analysis. Placental and PBL DNAs show germline configuration of the JH locus. The probe did not cross-hybridize with SCID DNA which is present in all tumor samples. With the exception of tumor 6, clear oligoclonal patterns were seen. Exposure time was 15 days. (B) BamNJ analysis. A duplicate membrane was obtained as previously described. Note that tumor 6, although apparently monoclonal by JH analysis, showed more than one episomal DNA form. Reduced exposure times revealed that 6 and 5 different circular forms were present in tumors 8 and 9, respectively. Linear DNA was present in all the samples analyzed. It should be noted that a tumor may be considered polyclonal by BamNJ analysis due to superinfection and generation of replicative intermediates during viral replication, but a monoclonal tumor by JH rearrangements. However, the presence of at least two B-cell clones in tumor 6 was confirmed by the presence of IgG and IgM and both k and l light chains in culture supernatants.

Fig. 9. Western blot analysis of primary hu-PBL-SCID tumors, tumor lines established from hu-PBL-SCID mice, and LCL-derived tumors. Ramos and Daudi are EBV-negative and EBV-positive Burkitt's lymphoma lines, respectively. RG is an in vitro B95-8-transformed LCL. Tumor lines carry the same number as the tumor from which they were established. Human and mouse cellular proteins detected in B, C, and D are due to nonspecific binding of secondary antibodies. (A) A representative number of tumor lines were examined for EBNA-1 expression with serum DEM. Note that tumor lines from each donor are characterized by a particular molecular weight EBNA-1 protein. This diversity is due to variability in the length of the glycine-alanine region of EBNA-1 and has been useful in distinguishing between different EBV isolates (32). (B) EBNA-2 and LMP-1 were simultaneously detected in tumor lines with PE-2 and S-12 monoclonal antibodies, respectively. EBNA-2 was detected as a Mr ~90,000 protein; however, an extra band migrating as an ~Mr ~86,000 protein was seen on occasion. LMP-1 was detected as a ~Mr ~60,000 protein in all samples analyzed with the exception of tumor lines derived from donor 4. (C) EBNA-2 expression in hu-PBL-SCID and LCL tumors. Representative data are shown. EBNA-2 was undetectable in the samples studied. (D) LMP-1 expression in hu-PBL-SCID and LCL tumors. LMP was detected in both types of tumors as a ~Mr ~60,000 protein.
outgrowth of latently infected B-cells (a one step transforming mechanism) leading to late, monoclonal tumors; and virus replication and infection of bystander B-cells (a two step transforming mechanism) leading to more rapid appearance of polyclonal tumors. This hypothesis is consistent with a previous report showing that human B-cells are susceptible to EBV infection in the SCID mouse environment (31) and is supported by the isolation of infectious virus from the peritoneal cavity of tumor-bearing mice. Whether viral replication occurs in human EBV-associated B-cell lymphomas, leading to a possible two step transforming mechanism, remains controversial. While Katz et al. reported the presence of linear DNA in ~40% of EBV-associated lymphomas in immunosuppressed patients (21), no evidence of viral replication was detected in two other studies (7, 8).

Characterization of B-cell differentiation and activation markers as well as EBV latent antigens confirmed that hu-PBL-SCID tumors resemble, but are not identical to, in vitro transformed LCL. We found that freshly isolated tumors derived from all donors expressed less CD23 and EBNA-2 than their corresponding in vitro passaged cell line or B95-8 transformed LCL (Figs. 3 and 9C) and that transfer of LCL to SCID mice led to a reduction in CD23 and EBNA-2 expression. These observations suggest that CD23 and EBNA-2 expression are modulated in response to the environment in which the transformed B-cell resides. Since EBNA-2 is known to induce CD23 expression (37), their comodulation is not surprising. Normal levels of EBNA-2 expression have been reported in previous studies involving PTL (7, 8), lymphomas induced by experimental infection of cottontop tamarins (38), or hu-PBL-SCID tumors (16); however, lack of EBNA-2 expression evaluated by Western blot has been observed recently in certain cases of PTL.* The reduced expression of EBNA-2 may have important implications, inasmuch as it has been proposed as one of the main targets for cytotoxic T-cell activity (39).

We did not observe down-regulation of LMP-1 expression, which is surprising since EBNA-2 is thought to control LMP-1 (40). However, the sensitivity of LMP-1 detection by Western blot was higher than that for EBNA-2, suggesting that the signal seen for LMP-1 in hu-PBL-SCID tumors might have been derived from the few EBNA-2-expressing cells detected by indirect immunofluorescence. The expression of LMP-1 in the absence of EBNA-2, if correct, would not be unprecedented. It has been reported to occur in some cases of nasopharyngeal carcinoma (41) or EBV-associated Hodgkin’s disease (42). These observations on EBNA-2, LMP-1, and CD23 expression raise an important question: is the role of EBV latent gene expression in the generation of tumors in vivo different than its role in transformation in vitro?

In conclusion, we have demonstrated the existence of heterogeneity among healthy EBV-seropositive donors in their potential to generate EBV-associated B-cell lymphomas in the hu-PBL-SCID mouse model. This model system should permit studies to define risk factors for development of B-cell lymphomas in patients subjected to immunosuppressive therapy (17, 18) or with AIDS (43). It may prove useful to prospectively screen EBV-seropositive transplant recipients or bone marrow donors in order to evaluate the risk of posttransplant lymphoma development.

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Note Added in Proof

Additional experiments conducted recently confirm that primary tumors arising in hu-PBL-SCID mice have much reduced EBNA-2 and CD23 expression; however, transfer of in vitro transformed LCL to SCID mice results in intermediate levels of expression of both EBNA-2 and CD23, not the virtual absence implied by the Western blot data in Fig. 9C.

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Heterogeneity among Epstein-Barr Virus-seropositive Donors in the Generation of Immunoblastic B-Cell Lymphomas in SCID Mice Receiving Human Peripheral Blood Leukocyte Grafts


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