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Differential Epstein-Barr Virus Gene Expression in B-Cell Subsets Recovered from Lymphomas in SCID Mice after Transplantation of Human Peripheral Blood Lymphocytes†

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We have analyzed the human B-cell tumors that arise spontaneously in SCID mice who have been given transplants of peripheral blood lymphocytes from Epstein-Barr virus (EBV)-seropositive donors to determine if patterns of EBV gene expression are correlated with phenotypic changes in the tumor B cells. Tumor cells were separated into two B-cell subsets by cell sorting on the basis of differential coexpression of membrane CD23 and CD38. One subset showed intermediate levels of CD23 and CD38 expression ($CD23^{int}CD38^{int}$), while a second subset had low-level CD23 but high-level CD38 expression ($CD23^{lo}CD38^{hi}$). The $CD23^{int}CD38^{int}$ cells had a high proliferative index and secreted little immunoglobulin in vitro; the $CD23^{lo}CD38^{hi}$ cells had a low proliferative index and high-level immunoglobulin secretion. We next analyzed the sorted cells for viral transcripts associated with latency (EBNA-1, EBNA-2, and LMP-1) or lytic cycle replication (ZEBRA and gp350 envelope protein). Only latent cycle transcripts were found in $CD23^{int}CD38^{int}$ cells, whereas lytic cycle transcripts and transforming virus were present in the $CD23^{lo}CD38^{hi}$ cells. Finally, we generated short-term cell lines from the sorted $CD23^{int}CD38^{int}$ cells and transferred these cells to SCID recipients. The resulting secondary tumors were predominantly $CD23^{lo}CD38^{hi}$, suggesting that the $CD23^{int}CD38^{int}$ lymphoblastoid cells are precursors to the well-differentiated, plasmacytoid $CD23^{lo}CD38^{hi}$ cells. These observations are discussed in the context of a three-step model for EBV-associated lymphomagenesis in humans.

Epstein-Barr virus (EBV) is associated with B-cell lymphomas that develop during genetic (19, 24), acquired (10), or induced (9) immunodeficiency. This association has led to the proposal that EBV has a direct role in the pathogenesis of these diseases (16). The complex interaction between virus and host B cells has been analyzed by using EBV-immortalized lymphoblastoid cell lines (LCLs) generated in vitro and, more recently, in the B-cell lymphomas that develop after xenotransplantation of peripheral blood lymphocytes (PBLs) into SCID mice (3, 20, 25, 29). The patterns of EBV gene expression and B-cell differentiation differ between these in vitro and in vivo models for transformation, and the relevance of each model for the development of EBV-associated lymphomas in patients remains to be established.

LCLs proliferate indefinitely in culture, produce low levels of immunoglobulin (Ig), and resemble activated normal B cells in the expression of CD23, CD30, CD39, CD40, and CD70 and the adhesion molecules CD11a, CD18, and CD54 (2, 36). All EBV latent genes (Epstein-Barr nuclear antigen [EBNA]-1, -2, -3a, -3b, -3c, and -LP; latent membrane protein [LMP]-1, -2a, and -2b; and two untranslated RNA species [reviewed in reference 15]) are expressed in LCLs. Furthermore, a direct role for several of these viral proteins in B-cell transformation in vitro has been demonstrated (6, 14, 17, 33). A small percentage of cells within the LCLs reactivate lytic cycle infection. Treatment of LCLs with 12-*O*-tetradecanoylphorbol-13-acetate or *n*-butyrate, both of which induce B-cell differentiation, increases the frequency of cells entering the lytic cycle (18), suggesting a correlation between B-cell differentiation and activation of the lytic cycle. LCL cells transferred to SCID mice show a shift to a more plasmacytoid appearance, reduced CD23 expression, and increased CD38 expression, as well as a decrease in EBNA-2 and LMP-1 expression (28), further suggesting that the state of B-cell differentiation correlates with changes in viral gene expression. However, activation of lytic cycle EBV replication is not seen in these LCL-derived tumors.

The second model for EBV-associated lymphomagenesis is SCID mice given transplants of PBLs from EBV-seropositive donors. These human (hu)-PBL-SCID chimeras spontaneously develop oligoclonal B-cell tumors that closely resemble the phenotype of B-cell lymphomas observed in immunodeficient patients (3, 20, 22, 29). hu-PBL-SCID lymphomas exhibit low-level expression of EBNA-2 protein and variable expression of lytic cycle proteins (e.g., ZEBRA and early antigen complexes) and lytic cycle replication (25, 29). Furthermore, hu-PBL-SCID tumors show both lymphoblastoid and plasmacytoid cells (25, 26, 29).

EBV latent gene expression and B-cell activation antigens are detected in EBV-associated B-cell lymphomas or lymphoproliferative disease (LPD), suggesting to some that these tumors are the in vivo equivalent of LCLs (32, 38). However, a more detailed analysis of these tumors suggests that they are more similar to the hu-PBL-SCID lymphomas. For example, variable expression of CD23 is observed in EBV-associated B-cell lymphomas (23, 32) and a mixture of lymphoblastoid and plasmacytoid cells is often seen (7, 21, 27). In addition, low and variable expression of the EBNA-2 viral latent protein is observed in LPD (4, 8, 32), even when compared with LCLs generated from the same patient (4). Finally, while EBV lytic cycle replication is virtually undetectable in LCLs, there is

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considerable variability in the detection of viral lytic cycle genes or linear replicating genomes in LPD (4, 12, 13, 23, 32).

The heterogeneity observed in both the B-cell phenotype and EBV gene expression in EBV-associated B-cell lymphomas leaves unresolved the underlying mechanism of lymphomagenesis. Using the hu-PBL-SCID model, we show here that there are two predominant cell populations in EBV-induced B-cell tumors: a lymphoblastoid population expressing EBV latent genes and a plasmacytoid population expressing EBV lytic cycle genes. We also present evidence that the plasmacytoid cells originate from the lymphoblastoid cells. Our results suggest that distinct patterns of EBV gene expression coincide with distinct phases of B-cell differentiation and that this process culminates in plasmacytoid cells undergoing EBV lytic cycle replication.

MATERIALS AND METHODS

SCID mice and cells. Human PBLs were obtained from a healthy EBV-seropositive donor and were purified as described previously (25). Fifty $\times 10^6$ cells were injected intraperitoneally into eight C.B.17-*scid*/*scid* mice obtained from The Scripps Research Institute breeding colony. Tumors developed in all mice by 63 days postreconstitution. Recovered tumors were either fixed in 10% formalin for histology or dispersed into single-cell suspensions by mincing the tumor sample in RPMI 1640 and then passing the cells through a cell strainer (Falcon). Dead cells were eliminated by density gradient centrifugation with Ficoll-Hypaque (Pharmacia). The tumor cells were greater than 90% viable as determined by trypan blue dye exclusion. The Akata cell line (31) was obtained from M. J. Cannon (The Scripps Research Institute), and JW-LCL was derived by infection of PBLs with the B95-8 strain of EBV (25). Both lines were maintained in RPMI 1640 supplemented with 10% fetal calf serum, 2 mM glutamine, and antibiotics.

Immunofluorescent staining and flow cytofluorimetric analysis and sorting. For analysis of cell surface molecules, the following monoclonal antibodies (MAbs) were used in this study: fluorescein isothiocyanate (FITC)-conjugated anti-CD10 (Becton Dickinson, San Jose, Calif.), FITC-conjugated anti-CD19 (Becton Dickinson), FITC-conjugated anti-CD23 (The Binding Site, La Jolla, Calif.), and phycoerythrin (PE)-conjugated anti-CD38 MAb (Becton Dickinson). Staining was performed by standard procedures (25). Stained cells were analyzed with a FACScan instrument by using the Lysis II program (Becton Dickinson). For cell sorting, tumor cells were stained with both FITC-conjugated anti-CD23 MAb and PE-conjugated anti-CD38 MAb and were separated with a FACS-Star cell sorter (Becton Dickinson). The flow cytofluorimetric sorting and phenotypic analysis were performed with two separate tumor preparations.

Cell culture. Sorted cells, unfractionated tumor cells, or LCL cells were placed in triplicate cultures in 96-well plates and cultured for 48 h. Medium was analyzed for Ig levels by enzyme-linked immunosorbent assay as described previously (25). Cultures were then pulsed for 4 h with 1 μCi of [^{3}H]thymidine (Amersham) and were harvested onto glass fiber filters and processed for liquid scintillation spectroscopy. A separate culture was established in 48-well plates at a density of 2×10^5 cells per well to determine the ability of sorted tumor cells to establish cell lines and to produce virus.

RNA extraction and ribonuclease protection assay. RNA was extracted according to the methods of Chomczynski and Sacchi (5). The EBNA-2, LMP-1, CD23, and ribosomal protein (rp) L32 riboprobes used for the ribonuclease protection assay have been described previously (28). The EBNA-1 riboprobe (nucleotides 890 to 1095 [30]) was made as described for the other protection assay clones (28). The ribonuclease protection assay was done exactly as described previously (11), except that only RNase T1 was used to digest the RNA duplexes.

PCR analysis of lytic cycle transcripts. Total RNA from 10^5 cells was used for random primed cDNA synthesis with the GeneAmp Kit (Perkin-Elmer Cetus). After cDNA synthesis, PCR buffer, $MgCl_2$ (2.5 mM), and primers specific for gp350 or ZEBRA were added to the cDNA at 50 pmol each. The samples were heated to 94°C for 4 min and then amplified for 30 cycles of 30 s at 90°C, 30 s at 55°C, and 30 s at 72°C. Aliquots of each reaction mixture were analyzed on 2.0% agarose gels in 0.04 Tris acetate–0.001 M EDTA, and the gels were transferred to nitrocellulose and probed with a ^{32}P-labeled oligonucleotide probe. The sequences of the gp350 primers were 5′-CACAGGCCCCACTGTATC and 5′-GAGGTGGAGCTGGTCATG (nucleotides 2402 to 2544 [GenBank accession number M10593]), and that of the probe was 5′-AGATGGACTTGGTGTCAC. The sequences of the ZEBRA primers were 5′-CCCAGTCTCGCACATAAC and 5′-GCCACCCGATTCTTGTATC, and that of the probe was 5′-CTAGTTCAGAATCGCATTCC (nucleotides 1232 to 1502) (1).

In situ hybridization. A probe specific for gp350 was generated by cloning the reverse transcriptase PCR product (described above) into pGem4 (Promega, Madison, Wis.). Antisense transcripts were generated in vitro in the presence of digoxigenin-UTP according to the manufacturer's protocol (Boehringer Mannheim, Indianapolis, Ind.). Tumor cells, cells expressing a low level of CD23 and a high level of CD38 ($CD23^{lo}CD38^{hi}$), and the H9 human T-cell line (negative control) were deposited on silanated slides (Digene Diagnostics, Silver Spring, Md.) and allowed to air dry. Cells were fixed in 4% paraformaldehyde. Hybridization was performed at 45°C overnight in 50% formamide and 10% dextran. Alkaline phosphatase-labeled anti-digoxigenin antibody (Boehringer Mannheim) was used to detect cells hybridizing with the probe, and nuclear fast red was used to counterstain the cells.

RESULTS

Characterization of hu-PBL-SCID tumors. We compared the cell surface phenotype of hu-PBL-SCID lymphomas with that of an LCL (JW-LCL) and with those of a Burkitt's lymphoma cell line (Akata). Cells were stained with MAbs for four different B-cell markers: CD10, CD19, CD23, and CD38. A representative flow cytofluorimetric analysis is shown in Fig. 1. All of the tumor cells expressed CD19, confirming their human B-cell origin. In addition, tumor cells expressed intermediate levels of CD23, a B-cell activation marker, and high levels of CD38, a marker found on pre-B cells and plasma cells (34). In comparison, JW-LCL cells expressed high levels of CD23 and low to intermediate levels of CD38 and were positive for CD19 expression. Significant CD10 expression was detected only in the Akata cell line. Akata cells also expressed high levels of CD38 but very low levels of CD23. These results show that the hu-PBL-SCID tumors are not identical to LCLs or to the Akata Burkitt's lymphoma cell line on the basis of cell surface phenotype.

hu-PBL-SCID lymphomas are composed of at least two phenotypically distinct B-cell subsets. We have shown that coexpression of membrane CD23 and CD38 can be used to distinguish LCLs cultured in vitro from LCL-derived tumors after transfer of LCLs into SCID mice (28). To analyze the coexpression of CD23 and CD38 on spontaneous hu-PBL-SCID tumors, the tumor cells were stained with FITC anti-CD23 and PE anti-CD38 and flow cytofluorimetric analysis was performed. The data show that 41% of the tumor cells expressed intermediate levels of both CD23 and CD38 ($CD23^{int}CD38^{int}$), while 54% of the tumor cells expressed high levels of CD38 and low levels of CD23 ($CD23^{lo}CD38^{hi}$) (Fig. 2). The two tumor cell populations were easily separable by cell sorting: a typical reanalysis of isolated $CD23^{int}CD38^{int}$ and $CD23^{lo}CD38^{hi}$ cells is shown in Fig. 2.

Proliferative capacity and Ig secretion segregate with B-cell phenotype. To determine if the differences in cell surface phenotype extended to other phenotypic differences, the two populations of sorted B cells were placed in culture and were analyzed for proliferation and Ig secretion (Table 1). The $CD23^{int}CD38^{int}$ subset had a fourfold higher level of proliferation than the $CD23^{lo}CD38^{hi}$ subset. By contrast, the $CD23^{lo}CD38^{hi}$ subset had a 10-fold higher level of Ig secretion than the $CD23^{int}CD38^{int}$ subset. By comparison, JW-LCL cells had a 1.4-fold higher level of proliferation and an 18-fold reduction in Ig secretion relative to the $CD23^{int}CD38^{int}$ subset. The sorted populations were deposited on microscope slides, stained, and examined by light microscopy, which confirmed the enrichment of lymphoblastoid cells in the $CD23^{int}CD38^{int}$ fraction and the enrichment of plasmacytoid cells in the $CD23^{lo}CD38^{hi}$ fraction. These results clearly demonstrate that there are two predominant B-cell populations in the hu-PBL-SCID lymphomas which are phenotypically distinct as measured by both cell surface markers and biological activity.

Lineage relationship of tumor cell subsets. We attempted to propagate the two sorted tumor cell subsets in vitro to determine if both subsets were able to generate cell lines. After 1 week of culture, clear morphologic differences were observed.

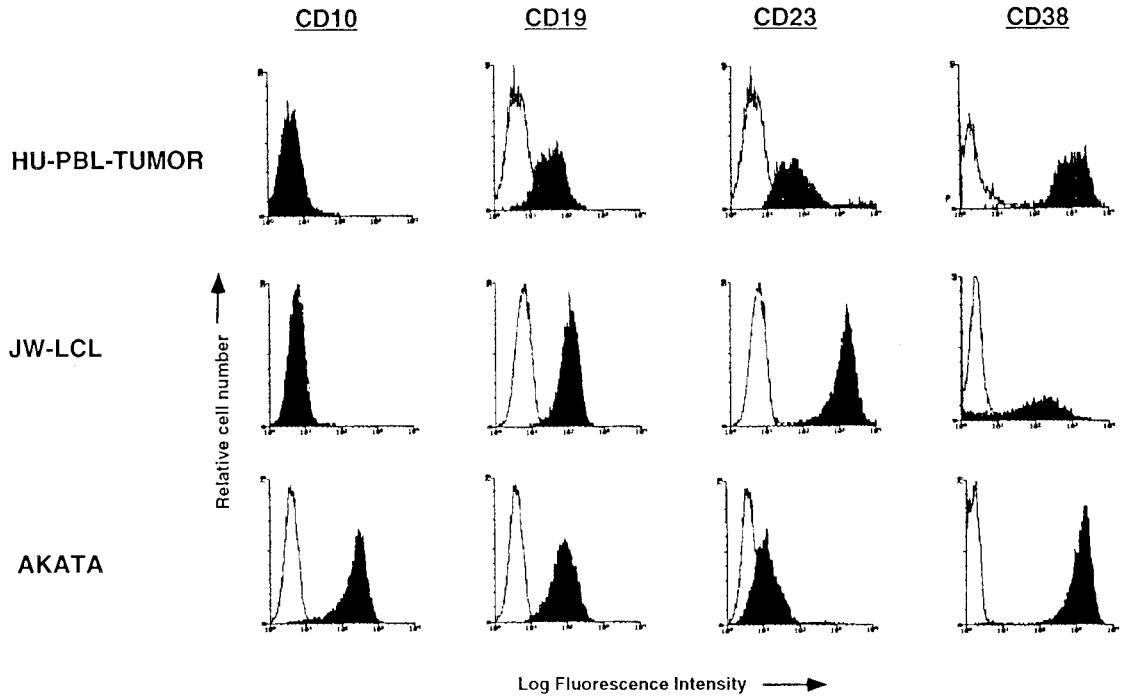

FIG. 1. Expression of B-cell surface markers. Tumor cells, the Akata cell line, and JW-LCL cells were stained with MAbs for CD10, CD19, CD23, and CD38 and subjected to flow cytofluorimetric analysis. Histogram plots of the relative fluorescence intensity are shown (solid histograms). The open histograms represent staining with the isotype-matched control antibody. The phenotype of the tumor shown was representative of all tumors analyzed ($n = 4$). Histograms were analyzed with the Lysis II program (Becton Dickinson).

The $CD23^{int}CD38^{int}$ cells formed large clumps of cells similar to LCL cells, whereas the $CD23^{lo}CD38^{hi}$ cells grew as single cells or small clumps of cells. In addition, after 2 weeks, the $CD23^{lo}CD38^{hi}$ cells showed relatively low viability (by trypan dye exclusion) and could not be expanded, as would be expected from their low rate of DNA synthesis (Table 1). In contrast, the $CD23^{int}CD38^{int}$ subset grew well in culture and we were able to generate a tumor cell line (TCL).

To determine if the $CD23^{int}CD38^{int}$ TCL was able to generate tumors in secondary SCID recipients, we injected the TCL into five SCID mice (3×10^6 cells per mouse). Tumor development was observed in all mice by 33 days after cell transfer. Both the parental TCL and the tumors derived from the TCL were analyzed for coexpression of CD23 and CD38 (Fig. 3). We observed that, after 2 months in culture, the TCL had lost CD38 expression and had a further increase in CD23 expression and so now resembled the LCL phenotype (Fig. 1). However, after transfer of the TCL to SCID mice, the resulting tumor cells showed a substantial shift to $CD23^{lo}$ expression and had regained expression of CD38. This phenotype is similar to that of the original tumors (Fig. 2). These data suggest that the expression of CD23 and CD38 was influenced by growth in tissue culture versus growth in SCID mice, and that the $CD23^{int}CD38^{int}$ lymphoblastoid cells can differentiate into $CD23^{lo}CD38^{hi}$ plasmacytoid cells, even after prolonged growth in culture.

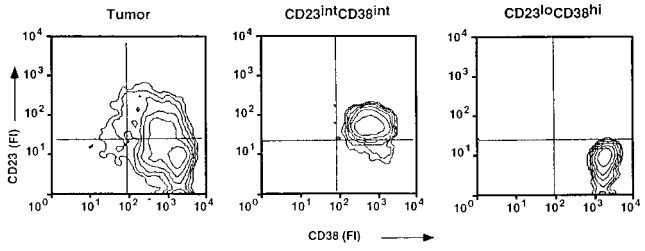

FIG. 2. Associated expression of CD23 and CD38 in hu-PBL-SCID tumors and tumor cell subsets. Tumor cells were stained with the FITC-conjugated anti-CD23 MAb and the PE-conjugated anti-CD38 MAb and were analyzed before and after cell sorting on the FACS-Star cell sorter. Two gates were set, and the reanalyses of the resulting populations ($CD23^{int}CD38^{int}$ and $CD23^{lo}CD38^{hi}$) are displayed as bivariant contour plots. Greater than 90% purity of the two sorted populations is indicated. Fl, fluorescence.

EBV gene expression segregates with the B-cell phenotype. To investigate if the phenotypic differences in tumor cell subsets correlated with differences in EBV gene expression, latent gene expression was measured in total RNA extracted from the sorted cell subsets and compared with RNA from unfractionated tumor cells and from JW-LCL cells. We developed a multiprobe ribonuclease protection assay as a quantitative measurement of mRNA for the viral latent genes LMP-1, EBNA-1, and EBNA-2 and for the cellular genes CD23 and rpL32 (Fig. 4A). A clear segregation in latent gene expression was observed; latent transcripts were detected only in the $CD23^{int}CD38^{int}$ subset, as was CD23 mRNA, even though the tumor cell and tumor cell subsets had equivalent

TABLE 1. Phenotype analysis of tumor cell subsets

Cell subset	Proliferation (10^3 cpm)	Ig (μg/ml)
LCL	50.7	0.21
Tumor	18.2	1.7
$CD23^{int}CD38^{int}$	35.4	3.7
$CD23^{lo}CD38^{hi}$	0.8	32.6

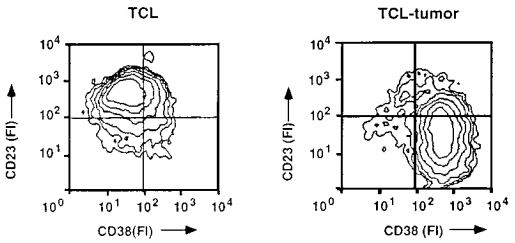

FIG. 3. Coexpression of CD23 and CD38 in a TCL and secondary tumors after transfer into SCID mice. The TCL was derived from the $CD23^{int}CD38^{int}$ subset after growth in culture for 2 months, and then TCL cells were transferred to SCID mice. A representative pattern is shown for one of five tumors analyzed and is displayed as a bivariant contour plot. Fl, fluorescence.

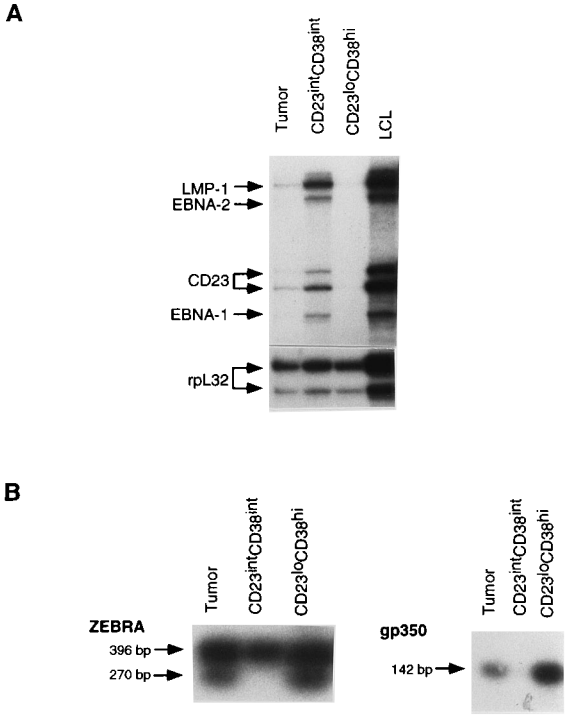

FIG. 4. Viral and cellular gene expression in hu-PBL-SCID tumor and tumor cell subsets. (A) A multiprobe ribonuclease protection assay was performed to detect viral EBNA-1, EBNA-2, and LMP-1 mRNA and cellular CD23 and rpL32 in tumor cells and sorted tumor cell subsets. RNA from 8×10^5 cells was analyzed in each track. The autoradiogram was exposed for 24 h. (B) PCR analysis for gp350 expression and ZEBRA expression. cDNA from tumors or tumor subsets was amplified by PCR with primers specific for gp350 or ZEBRA. The PCR product was run on agarose gels and analyzed for specific PCR products by blot hybridization with a ^{32}P-labeled oligonucleotide probe internal to the PCR product. Two bands are seen after amplification with the ZEBRA primers. The 396-bp band corresponds to the size of the unspliced transcript and most likely represents DNA contamination. The 270-bp band corresponds to the size of the spliced mRNA.

levels of rpL32 mRNA (a housekeeping gene transcript). The level of RNA expression in the unfractionated tumor relative to that in JW-LCL cells was reduced for both cellular and viral genes. Total RNA extracted quantitatively from equivalent cell numbers was used in this assay, thus suggesting that the mRNA per cell from the tumor was reduced relative to that from JW-LCL cells. These data indicate that the only detectable source of latent gene expression is the $CD23^{int}CD38^{int}$ subset.

There are two possible reasons for the undetectable level of EBV latent gene expression in the $CD23^{lo}CD38^{hi}$ subset: either EBV has entered the lytic cycle, or the cells are not EBV positive. We analyzed lytic gene expression in the tumor cell subsets by using reverse transcriptase PCR to measure mRNA for ZEBRA, an immediate-early lytic cycle protein, and for gp350, the major envelope glycoprotein. Both ZEBRA and gp350 mRNA were detected in the $CD23^{lo}CD38^{hi}$ subset and the unfractionated tumor cells, but not in the $CD23^{int}CD38^{int}$ population (Fig. 4B). These data show that EBV has been reactivated to the lytic cycle in at least some cells within the $CD23^{lo}CD38^{hi}$ subset. To determine the percentage of cells in the $CD23^{lo}CD38^{hi}$ subset that had reactivated EBV lytic cycle replication, sorted $CD23^{lo}CD38^{hi}$ cells were analyzed for gp350 mRNA expression by in situ hybridization. Eighty-seven percent (174 of 200 cells examined) of the $CD23^{lo}CD38^{hi}$ cells gave a strong hybridization signal (data not shown), thus suggesting that EBV had entered the lytic cycle in the majority of cells in the $CD23^{lo}CD38^{hi}$ subset.

To confirm the production of transforming virus, sorted subsets were cultured for 7 days, after which the supernatant was removed and analyzed for the presence of transforming virus by addition to cultures of EBV-seronegative PBLs. Cultures were monitored for 6 weeks for the development of growing foci of lymphoblastoid cells. After only 2 weeks, transformants were obvious in the wells that received the $CD23^{lo}CD38^{hi}$ subset supernatant, whereas transformants were not observed in cultures receiving the $CD23^{int}CD38^{int}$ subset supernatant.

DISCUSSION

In this report, we have shown that EBV-associated human B-cell lymphomas arising in hu-PBL-SCID mice are composed of two distinct EBV-infected B-cell populations: $CD23^{lo}$ $CD38^{hi}$ and $CD23^{int}CD38^{int}$ cells. Analysis of these subsets demonstrated a correlation of lytic viral gene expression with a more differentiated B-cell phenotype (high-level Ig secretion, low-level Ig proliferation, low-level membrane CD23, and high-level membrane CD38 expression), while viral latent gene expression was found in cells with a lymphoblastoid phenotype (low-level Ig secretion, high-level proliferation, and intermediate-level membrane CD23 and CD38 expression). These data suggest that the only source of latent gene expression is in the $CD23^{int}CD38^{int}$ subset. The reduced levels of EBNA-2 protein relative to LCL observed in previous reports on LPD biopsies and hu-PBL-SCID tumors (4, 25) could be attributed to the analysis of protein from whole biopsies that would average EBNA-2 content from both subsets. However, comparison of mRNA expression in the lymphoblastoid $CD23^{int}$ $CD38^{int}$ subset with that of the LCLs showed a lower overall level of EBNA-1, EBNA-2, and LMP-1 transcripts and lower levels of CD23 transcripts, suggesting that the lymphoblastoid phenotype found in vivo is not the equivalent of LCLs in vitro. This same reduction in latent gene expression has been observed after transfer of LCLs to SCID mice (28). This reduced expression of target antigens for cytotoxic T cells could be a mechanism of escape from virus-specific immune surveillance, especially in a host with a weakened response due to disease or immune suppression.

The absence of EBV latent gene expression in the $CD23^{lo}CD38^{hi}$ tumor cells is surprising, because these cells are presumed to be derived from the latently infected lymphoblastoid cells. Although the RNase protection assay might fail to detect very low levels of EBNA-1, EBNA-2, and LMP-1 mRNA expression, this result implies either that virtually all of the $CD23^{lo}CD38^{hi}$ plasmacytoid cells have entered lytic cycle

viral replication or that loss of the viral episome has occurred. The high frequency of cells expressing gp350 mRNA in the $CD23^{lo}CD38^{hi}$ plasmacytoid cells suggests that EBV has entered the lytic cycle in the majority of plasmacytoid cells. The association of lytic gene expression with the terminally differentiated B cells is similar to observations that EBV lytic replication is restricted to terminally differentiated epithelial cells (39).

We also found that the purified $CD23^{int}CD38^{int}$ subset shifts phenotype to $CD23^{lo}CD38^{hi}$ after short-term growth in culture and, after transfer and growth in SCID mice, shifts to a $CD23^{lo}CD38^{hi}$ phenotype. It remains to be determined what signals are driving these phenotypic shifts and whether they are acting on viral or cellular gene expression. The outgrowth of the $CD23^{int}CD38^{int}$ TCL subset, but not the $CD23^{lo}CD38^{hi}$ subset, supports the proposal by Cen et al. (4) that outgrowth of LCLs derived from LPD lesions represents the in vitro selection of cell subpopulations and is not representative of the cell types that are found within lymphomas. Both the phenotype and growth characteristics of EBV-transformed B cells thus are influenced by the environment in which they are maintained.

Our results provide potential explanations for the unresolved question regarding EBV lytic cycle replication and its association with B-cell lymphomas and LPDs. EBV latent genes are sufficient for transformation of B cells in vitro (6, 14, 17, 33), so lytic cycle replication should not be expected to be essential for tumor appearance, but virus production by reactivated B cells could shorten tumor latency and increase polyclonality by infection and transformation of previously uninfected bystander B cells. We have previously observed that lytic replication in hu-PBL-SCID tumors correlates only with PBL donors who have a high frequency of tumors with short latency after xenotransplantation (25). By contrast, those tumors that developed late after transplantation and at a low frequency rarely showed evidence of lytic replication. We now show that only a subset of cells (the $CD23^{lo}CD38^{hi}$ subset) within the tumors are lytically infected. Thus, the variability in detecting lytic cycle replication in human LPDs and lymphomas (4, 12, 13, 23, 32) might be influenced by the time of sampling (e.g., duration of immunosuppression) and the cellular composition of the tumor sample.

Spontaneous outgrowth of LCLs in vitro from PBLs was shown to occur by a two-step mechanism: reactivation from a latent infection to a lytic infection followed by infection of bystander B cells and transformation of these cells (37). Rowe et al. (29) proposed that a similar mechanism is occurring in vivo after immunosuppression of the host. Our results support this model and extend it by demonstrating that the reactivation of lytic cycle replication occurs only within a subpopulation of more differentiated tumor cells. Taken together, these results support a model for EBV-induced lymphomagenesis in which the following self-perpetuating series of events occurs: (i) proliferation of latently infected B lymphoblasts; (ii) differentiation to plasmacytoid cells with reactivation of lytic cycle infection and release of new virus; and (iii) infection of bystander B cells, some of which then proliferate as latently infected lymphoblasts. Most of the spontaneous tumors are oligoclonal, and rare monoclonal tumors have been observed after a long latency period (25), so the rate of generation of secondary transformants must be relatively low. The role of immunosuppression in this process could be to allow proliferation of latently infected lymphoblastoid cells (suppression of T cells recognizing EBV latent antigens) and to blunt immune responses to reactivated lytic cycle replication. The positive role of CD4 T cells in supporting tumor formation in hu-PBL-SCID mice (35) could result from their support of B-cell differentiation, which would in turn lead to activation of lytic cycle replication in plasmacytoid cells. The observations that posttransplant LPDs may resolve if immunosuppression is halted suggest that a competent immune response to both latent and lytic cycle EBV antigens can reverse this process. Finally, aggressive antiviral therapy directed at EBV replication may reduce the incidence of posttransplant lymphomas.

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