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Cell surface phenotyping and cytokine production of Epstein–Barr Virus (EBV)-transformed lymphoblastoid cell lines (LCLs)

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Abstract

Epstein–Barr Virus-transformed B lymphoblastoid cell lines (EBV-LCLs) are routinely used for the in vitro expansion of T cells. However, these cell lines are reported to produce the cytokine IL-10, which is inhibitory for T cells. We, therefore, characterized a panel of 37 EBV-LCLs for a variety of cell surface markers, for secretion of various cytokines including IL-10 and for immunoglobulin production. These cell lines were derived from normal donors or patients with nonsmall cell lung cancer, acute myelogenous leukemia, melanoma or colon cancer. Overall, 26 lines were positive for CD19 and CD20, and 11 were negative for both. All of the lines were strongly HLA-DR+, while CD40 expression was variable. Twenty-four (65%) were both CD23+ and secreted immunoglobulin, and 33 expressed κ and/or λ light chains. Additionally, all of the EBV-LCLs were negative for T cell (CD3), NK cell (CD16, CD56), monocyte (CD14) and granulocyte (CD66b) surface markers. Some level of IL-10, IL-6, IL-12p40 and TNF-α cytokine production was detected in 33, 18, 19 and 12 EBV-LCLs, respectively. Together, these data reflect the heterogeneity of EBV-LCLs, which cautions their use nondiscriminately in various immunologic assays.

Keywords: EBV; Cytokine; Feeder cells; Immunotherapy; NSCLC

1. Introduction

The Epstein–Barr Virus (EBV) is associated with a variety of human diseases, including infectious mononucleosis, and numerous malignancies, such as Burkitt's lymphoma, nasopharyngeal carcinoma, Hodgkin's lymphoma and lymphoproliferative disorders arising in immunocompromised patients (Baumforth et al., 1999). In addition to its clinical impact, this lymphotropic virus is routinely used in the laboratory

Abbreviations: EBV, Epstein–Barr virus; LCLs, lymphoblastoid cell lines; PBMC, peripheral blood mononuclear cells; NK cell, natural killer cell; TIL, tumor-infiltrating lymphocytes; NSCLC, nonsmall cell lung cancer; AML, acute myelogenous leukemia; BARTs, *Bam*HI-A rightward transcripts; RT-PCR, reverse transcription-polymerase chain reaction; bp, base pair; FACS, fluorescence-activated cell sorting; Ig, immunoglobulin.

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setting to transform B lymphocytes and generate B lymphoblastoid cell lines (LCLs) (Neitzel, 1986). Most immortalized B cell lines are derived from either normal or patient peripheral blood mononuclear cells (PBMCs), although cord blood (Schneider and zur Hausen, 1975; Revoltella et al., 2000) and fetal hematopoietic tissues (Ernberg et al., 1987; Hui et al., 1989; Muraguchi et al., 1992; Ichigi et al., 1993) have also been utilized. Whereas EBV is etiologically associated with some human tumors, it is believed the virus promotes rather than causes tumor development (Anagnostopoulos and Hummel, 1996). Moreover, LCLs themselves are not tumorigenic (Lam and Crawford, 1991).

Innumerable studies have examined cell surface markers on EBV-LCLs. One report on five B cell lines derived from patients with posttransplant lymphoproliferative disease identified a phenotype of CD19+, CD21+, CD22+, CD43+, CD77+ and CD10 − (Randhawa et al., 1997). Four spontaneous LCLs transformed in vivo from patients with EBV-associated lymphoproliferative disorder were determined to be CD19+, CD21+, CD23+, CD38+ and CD40+ (Beatty et al., 1997). A comparison between a parental EBV-LCL and a human–mouse heterohybridoma secreting a human monoclonal antibody found only the parental cell line was CD23+ (Sa'adu et al., 1992). Another group derived two clones from an EBV-transformed B cell line. In addition to exhibiting different growth characteristics, one was strongly CD23+ and weakly HLA-DR+, while the other was negative for both antigens (Warrington et al., 1989).

Autocrine growth loops have been identified in studies examining cytokine production by EBV-LCLs, primarily through the use of neutralizing antibodies. IL-6 (Yokoi et al., 1990; Tosato et al., 1990; Tanner and Tosato, 1992) and TNF-β (or lymphotoxin) (Estrov et al., 1993) have been reported to act as autocrine growth factors for EBV-LCLs. A report on six monoclonal EBV-B cell lines found 4/6 produced IL-6 and 6/6 produced TNF-β (Jochems et al., 1991). Another cytokine associated with the growth of EBV-LCLs is IL-10, which promotes humoral immunity but inhibits cellular immunity (Burdin et al., 1997). One group assessed cytokine gene expression in 16 LCLs utilizing an RNase protection assay to identify mRNA transcripts (Rochford et al., 1997). All of these LCLs had high levels of TGF-β1, TNF-α and TNF-β transcripts. Most expressed lower levels of IL-10, there was variable expression of IL-6, IL-12p35, IL-12p40 and IFN-γ, and no IL-4 or GM-CSF mRNA was detected. The authors identified IL-10, TNF-α and TNF-β as candidate autocrine factors for EBV-LCL growth. Another report identified IL-6 and IL-10 production by 4/4 spontaneous LCLs, and determined IL-10 served as an autocrine factor (Beatty et al., 1997).

EBV-LCLs have served a variety of purposes in immunological studies. They have been used to generate heterohybridoma antibody-producing cell lines (Garzelli et al., 1986; Wallace et al., 1990; Fizil et al., 1996). A recent study constructed a targeted human antibody library from LCL clones for phage display selection of antigen-specific Fabs (Kempf et al., 2001). The fusion of tumor cell lines with EBV-LCLs produced stable hybrids with an enhanced ability to stimulate primary allogeneic T cell responses in vitro (Dunnion et al., 1999). Additionally, EBV-LCLs are often utilized as feeder cells for in vitro T cell or natural killer (NK) cell expansions. Using irradiated EBV-LCLs and allogeneic lymphocytes as feeder cells, fresh human serum and leucoagglutinin resulted in the expansion of single cells up to 10^9 (Van de Griend et al., 1984). This method did not require continual exogenous IL-2 as a medium supplement and permitted expansion for up to 60 generations in 2 months (Van de Griend and Bolhuis, 1984). The authors noted that some EBV-LCLs performed better than others. NK cells cultured with an EBV-LCL yielded increases in proliferation, purity and cytolytic activity (Rabinowich et al., 1991). Tumor-infiltrating lymphocytes (TIL) were cloned and expanded from a patient with cervical carcinoma (Hilders et al., 1994). TIL derived from thyroid tumors were efficiently expanded only in the presence of allogeneic EBV-LCL feeder cells (Lee et al., 1996). Our laboratory utilizes this general method to expand CTL and TIL from nonsmall cell lung cancer (NSCLC) patients. Occasionally, we experience limited success with our expansions. Therefore, we sought to characterize a panel of 37 EBV-LCLs that we can employ in these experiments to be able to determine which characteristics might help identify the best EBV-LCL feeder cells for T cell expansions.

2. Materials and methods

2.1. Generation and maintenance of EBV-transformed LCLs

Our EBV-transformed LCLs were cultured in complete medium consisting of RPMI-1640 (GIBCO/BRL, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Paragon Biotech, Baltimore, MD, USA), and 100 μM nonessential amino acids, 1 mM sodium pyruvate, 2 mM L-glutamine, 20 mM HEPES, 1.25 mg/l fungizone, 10 mg/l gentamicin (all from BioWhitaker, Walkersville, MD, USA) and 2.5 μg/ml M-plasmocin (InvivoGen, San Diego, CA, USA). Cells were grown at 37 °C in humidified air with 5% CO_2 in log phase. All EBV-transformed cell lines were maintained mycoplasma-free. For all assays, cell via-bility was >90%.

The EBV-transformed marmoset cell line B95-8 (Miller and Lipman, 1973) was grown to confluency, and infectious culture supernatants were harvested and stored at −80 °C until needed. Normal donor or patient samples of peripheral blood were separated by Ficoll–Hypaque gradient centrifugation to recover mononuclear cells (PBMC). Ten million PBMCs were added to 5 ml of B95-8 supernatant in a culture flask. Following a 1-h incubation at 37 °C, 5 ml of complete medium and 5 μg of cyclosporin A (Anderson and Gusella, 1984) were added. The cultures were incubated for 14–21 days until clumps were visible and the medium was yellow. The cultures were continually expanded until day 30 and then frozen down in FBS with 10% DMSO. Our rate of success at generating EBV-transformed cell lines from PBMC is 100%.

The PBMC donor histologies used to generate the EBV cell lines include: 18 patients with nonsmall cell lung cancer (NCI-H1650, UKY-29, UKY-53, UKY-76, UKY-78, UKY-79, UKY-80, UKY-81, UKY-82, UKY-84, UKY-85, UKY-90, UKY-96, UKY-97, UKY-98, UKY-99, UKY-108, UKY-109), eight patients with acute myelogenous leukemia (AML120398, AML011299, AML012799, AM013099, AML020199, AM020299, AML051399, AML091199), two melanoma patients (888-mel, UKY-MEL1), one colon cancer patient (UKY-CC1) and eight normal donors (UKY-38, UKY-77, UKY-2389, UKY-4638, UKY-4712, UKY-6949, UKY-7663, UKY-8467).

2.2. Reverse transcription-polymerase chain reaction (RT-PCR) for EBV BamHI-A rightward transcripts (BARTs)

EBV cell lines were cultured at 5×10^5 cells/ml in sterile T75 culture flasks in Complete-RPMI medium. Log phase cultures were harvested and total RNA was isolated using TRIzol reagent (GIBCO BRL, Gaithersburg, MD, USA). The integrity of the RNA was verified by gel electrophoresis and concentrations were calculated from OD_{260} readings. For analysis, 2 μg of total RNA were used for a one-step RT-PCR using the Access® RT-PCR system (Promega, Madison, WI, USA). The BART primers 5′ -CACGATGTCCTGGTCAGAGTG and 5′ -CCTTCGTATTGCAGTGTCTG (Zhang et al., 2001) were purchased from the MSAF Facility at the University of Kentucky, Lexington, KY, USA. These primers produce a 380-base pair (bp) product. A reverse transcription cycle of 48 °C for 45 min was followed by a PCR reaction as follows: 94 °C, 2 min; 45 cycles of 94 °C, 1.5 min; 57 °C, 1 min; 72 °C, 2 min; once at 72 °C, 15 min. PCR products were size fractionated by electrophoresis through a 1.5% agarose gel, visualized by ethidium bromide staining and UV transillumination and photographed.

2.3. Immunophenotyping and fluorescence-activated cell sorting (FACS) analysis

All cell lines were tested for cell surface antigen expression by direct immunofluorescence and flow cytometric analysis. The antibodies were conjugated with either fluorescein isothiocyanate (FITC) or phycoerythrin (PE) and included IgG_1 isotype controls (either FITC or PE), CD19-PE, CD20-FITC, κ light chain-FITC, λ light chain-FITC, CD40-FITC, HLA-DR-PE (MHC Class II), CD23-PE, CD3-PE (pan T cell), CD16-FITC (NK cell), CD56-PE (NK cell), CD14-PE (monocyte) and CD66b-FITC (granulocyte) (Becton Dickinson, San Jose, CA, USA). Briefly, $1-2 \times 10^6$ cells were pelleted in 12 × 75 tubes and washed twice in cold FACS buffer (PBS with 0.5% FBS, 0.03% sodium azide). Cells were resuspended in 80 μl of FACS buffer and 10 μl of 1:1 antibody dilutions in PBS were added. This represents

half of the manufacturer's recommended concentration, but we have previously tested this amount of antibody and obtained identical results as with undiluted antibody. Cells were incubated for 30 min at 4 °C, washed twice in cold FACS buffer and fixed in 500 μl of 1% formaldehyde. Flow cytometric analyses were performed on a Becton Dickinson FACScan, and all values given are the percentage of cells that express the given cell surface antigen. Ten thousand events were counted for each parameter set examined. Control gates (LL or lower left quadrant) were set to include >96% of cells along both the *x*- and *y*-axes. A cell line is considered positive for a given cell surface antigen if 10% or more of the cells are stained.

2.4. ELISA assays for cytokine and immunoglobulin (Ig) production

Standard ELISA assays for cytokine were performed according to the manufacturers recommended protocol with OptEIA ELISA kits purchased from Pharmingen, San Diego, CA, USA. OD^{450} readings were obtained with a Titertek Multiskan Plus plate reader (ICN, Costa Mesa, CA, USA). Cells were seeded at a concentration of 10^6 cells/ml and culture supernatants were harvested after 24 h and stored at −20 °C until tested. In some instances, culture supernatants were diluted 1:10 to obtain OD^{450} readings in the range of the standard curve for accurate protein quantification. For immunoglobulin detection, 96 well immuno-sorp plates (Nalge Nunc International, Fisher Scientific, Atlanta, GA, USA) were coated overnight with 5 μg/ml goat-anti-human-IgM or -IgG (Sigma, St. Louis, MO, USA). Plates were washed, blocked for 1 h at 37 °C, washed and incubated with undiluted 7–10 day culture supernatants for 1 h at 37 °C. Human sera diluted 1:200 served as a positive control. Again plates were washed and incubated as above with alkaline phosphatase conjugated anti-IgM or anti-IgG (Sigma), washed and developed with p-nitrophenyl phosphate (Sigma). OD readings at 405 nm were obtained with a Perkin Elmer HTS 7000 bioassay plate reader (Norwalk, CT, USA). IgM or nonspecific IgG were considered present when OD readings were 3 standard deviations above the medium control value.

3. Results

3.1. Cell surface immunophenotyping

The EBV-LCLs were characterized for 12 hematopoietic cell surface markers by direct immunostaining and FACS analysis. Fig. 1 illustrates some of the representative flow cytometric patterns obtained. UKY-7663-EBV and UKY-29-EBV are positive for B cell markers CD19 and CD20, while UKY-77-EBV and UKY-80-EBV are negative (column A). A cell line is considered to be positive for a given surface marker if 10% or more of the cells are stained. Each pair of LCLs was derived from a normal donor and an NSCLC patient, respectively. Only UKY-7663-EBV expresses both κ (column B) and λ (column C) light chains, while UKY-29-EBV and UKY-80-EBV express neither, and UKY-77-EBV expresses only λ chains. In addition, each of these LCLs is CD3 − (column B) and HLA-DR+ (column C).

The entire EBV-LCL panel tested negative for the expression of T cell (CD3), NK cell (CD16, CD56), monocyte (CD14) and granulocyte (CD66b) cell surface markers (data not shown). The results obtained for seven B cell lineage markers are presented in Table 1. Twenty-six (70%) of the EBV-LCLs expressed both CD19 and CD20, and 11 (30%) were negative for both. Thirty-three (89%) of the EBV-LCLs were positive for κ and/or λ light chains. All of the cell lines had a high percentage of cells expressing HLA-DR Class II major histocompatibility antigens. In addition, variable percentages of CD40+ cells were detected, with the majority of lines (26/37) at 50% or greater, 3/37 between 20% and 50% and 8/37 at only 10–20%. Finally, 24 (65%) of the cell lines were positive for CD23. These data suggest the LCLs are of B cell lineage, although heterogeneous expression patterns for cell surface markers were observed. There also was no phenotype specifically associated with LCLs derived from either normal donors, or AML patients, or NSCLC patients.

3.2. Immunoglobulin production by EBV-LCLs

We tested 7–10-day culture supernatants from the EBV-LCL panel for secreted IgM and IgG in a qualitative ELISA assay and the results are presented on a scale of low (+), moderate (++), high (+++) or

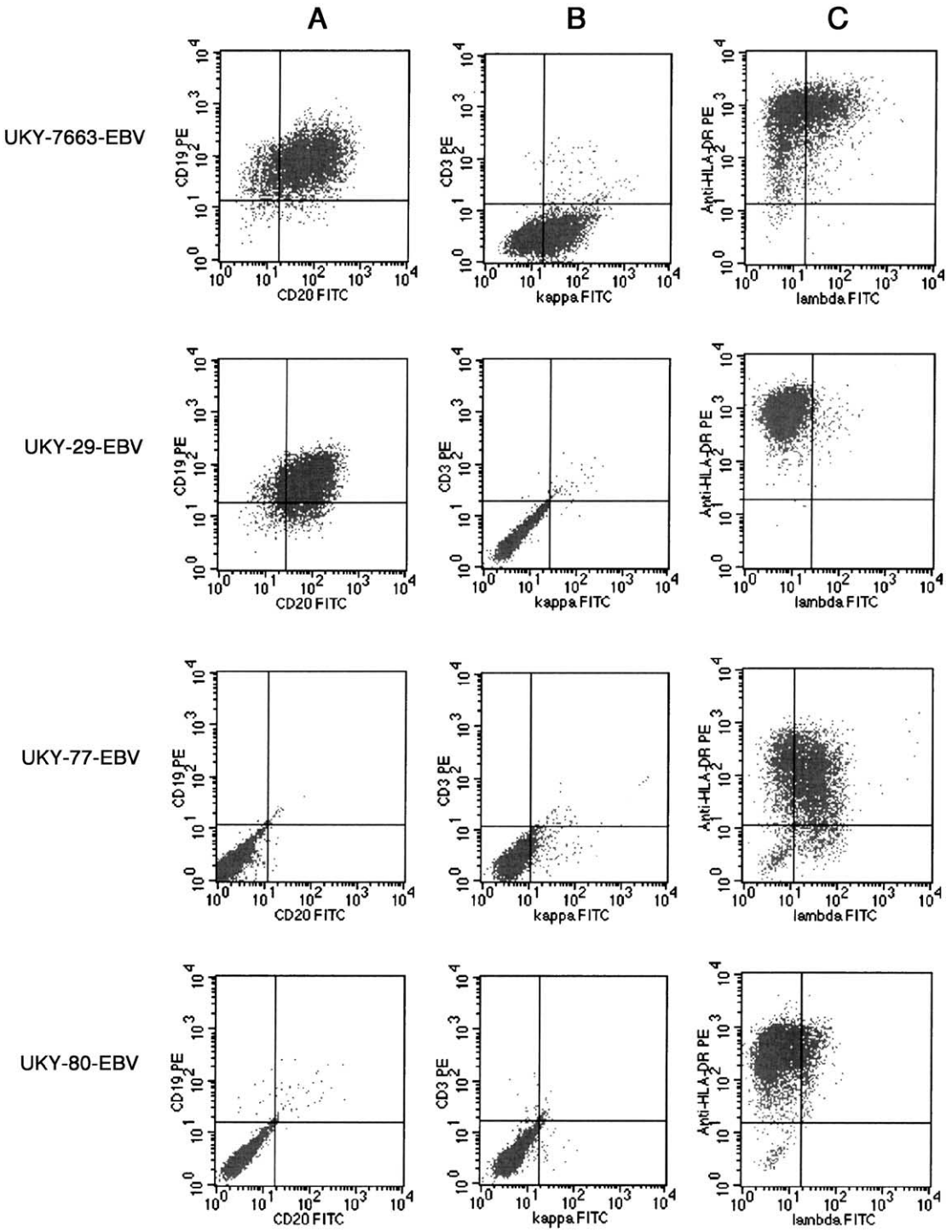

Fig. 1. Immunophenotyping by fluorescent-activated cell sorting (FACS). FACS analyses for CD19 and CD20 (column A), CD3 and κ light chains (column B) and HLA-DR and λ light chains (column C) are presented for four EBV-LCLs. UKY-7663-EBV and UKY-77-EBV were derived from normal donors, and UKY-29-EBV and UKY-80-EBV were derived from NSCLC patients.

Table 1
Immunophenotyping and cytokine and Ig production by EBV-LCLs

EBV line	Percentage of cells expressing[a]								Ig[b]		Cytokine production[c]			
	CD19	CD20	19/20	κ	λ	DR	CD40	CD23	IgM	IgG	IL-10	IL-6	IL-12p40	TNF-α
A														
AMLO20299	97	92	92	42	39	98	70	100	++++	++++	234	0	0	2
UKY-29	84	86	75	0	1	99	66	100	++++	+	204	7	95	0
UKY-38	98	94	93	35	53	99	79	99	++++	++++	388	0	28	0
UKY-8467	89	88	84	40	60	100	93	99	++++	++++	413	102	239	15
UKY-CC1	95	80	79	68	57	98	99	99	–	++++	557	20	0	62
NCI-H1650	96	69	67	10	2	98	87	99	+	++++	357	68	246	0
UKY-6949	96	85	85	13	52	98	64	98	++++	++++	323	30	0	4
AML051399	98	84	84	49	37	99	76	98	++++	++++	562	37	56	0
UKY-108	81	81	73	4	57	92	52	97	+	++++	397	0	115	0
UKY-99	98	63	63	15	43	99	68	96	++++	++++	745	6	0	0
888-mel	99	94	94	75	14	99	87	95	–	++++	539	20	31	0
UKY-2389	98	91	91	77	13	99	80	94	–	++++	569	0	0	0
UKY-82	53	44	43	42	9	99	72	94	+	++++	180	8	57	0
UKY-7663	87	87	85	66	37	98	99	93	+++	++++	678	11	436	18
UKY-98	86	80	74	10	20	97	76	93	++++	++++	847	209	1350	12
UKY-MEL1	95	81	81	67	47	99	87	92	++++	++++	845	128	1219	12
AML020199	84	85	79	52	40	98	73	91	++++	++++	233	10	0	0
AML013099	41	33	32	4	33	99	80	89	+	++++	252	0	0	0
UKY-81	95	73	73	68	4	99	99	86	–	++++	3680	0	35	0
UKY-109	90	56	54	13	12	98	78	82	++++	++	337	60	1306	21
UKY-53	98	82	82	13	36	99	82	68	–	++++	349	0	0	0
AML091199	85	78	78	47	40	99	55	57	++++	++++	1911	143	205	12
UKY-96	91	75	74	42	16	99	89	39	+++	++++	975	142	1720	22
UKY-79	19	17	15	6	12	99	44	34	++++	++++	53	0	152	0
B														
AML012799	49	49	44	1	51	99	10	1	+	–	1080	73	1497	17
UKY-97	33	18	14	27	32	99	23	3	+	+	301	843	1072	13
C														
AML120398	3	3	3	7	31	99	15	3	+	+	26	0	0	0
UKY-77	0	0	0	5	65	90	26	2	+	–	42	0	0	0
UKY-90	0	0	0	0	36	99	19	1	+	–	23	0	0	0
AML011299	0	0	0	8	89	99	14	1	+	–	21	0	0	0
UKY-84	0	0	0	0	10	99	16	0	+	–	13	0	0	0
UKY-76	0	0	0	2	44	98	10	0	+	–	24	0	0	0
UKY-80	0	0	0	0	0	97	10	0	–	+	13	0	3	0
D														
UKY-4638	0	0	0	1	17	95	19	1	+	–	0	0	0	0
UKY-4712	0	0	0	0	12	97	98	0	–	–	0	0	0	0
UKY-78	0	0	0	0	2	81	99	0	–	–	0	0	0	0
UKY-85	0	0	0	0	0	99	97	0	+	–	0	0	0	0

The results of cell surface marker immunophenotyping FACS analyses and ELISA assays for immunoglobulin and cytokine production for the EBV-LCL panel are presented. Group A consists of the 24 CD23+ cell lines. Group B contains the 2 CD23 – cell lines that produce significant amounts of IL-10, IL-6 and IL-12p40. The 7 CD23 – , CD19 – , CD20 – cell lines in Group C produce a low level of IL-10. The four CD23 – , CD19 – , CD20 – cell lines in Group D produce no cytokines and minimal Ig.

[a] Percentages of 10 or higher are considered positive.

[b] The scale for OD^{450} readings: + = 0.01 – 0.2, ++ = 0.2 – 0.4, +++ = 0.4 – 0.6, ++++ => 0.6.

[c] All results are expressed as pg/ml per 10^6 cells.

very high (++++) in Table 1. Twenty-four (65%) of our LCLs secreted significant amounts of immunoglobulin. Of these, 14 produced at least moderate (++) levels of both IgM and IgG, nine yielded very high signals (++++) for IgG and one (UKY-29-EBV) produced only (++++) IgM. Minimal to no immunoglobulin secretion was detected for 13 EBV-LCLs (Table 1, Groups B, C and D). Again there was no correlation identified with EBV-LCLs derived from normal donors, or AML or NSCLC patients. However, there was a strong positive correlation between CD23 expression and the secretion of very high (i.e. ++++) levels of immunoglobulin.

3.3. Cytokine production by EBV-LCLs

We also examined cytokine production by the EBV-LCLs in supernatants after a 24-h culture period. The results of these analyses are also presented in Table 1. In the majority of EBV-LCLs (34/37), measurable amounts ranging from 13 pg/ml to 3.7 ng/ml of the T cell inhibitory cytokine IL-10 were produced. IL-6 (6–843 pg/ml) was detected in the supernatants of 18/37 (49%) of the lines. While no IL-12 p70 was detected (data not shown), production of IL-12p40 was highly variable in 19 (51%) of the EBV-LCLs, ranging from 28 pg/ml to 1.7 ng/ml. TNF-α levels ranged from 2 to 62 pg/ml in 12 (32%) of the EBV-LCLs. Significant levels of GM-CSF, 70 and 194 pg/ml, were detected in the supernatants of AML091199-EBV and UKY-97-EBV, respectively (data not shown). None of the lines produced any detectable IFN-γ, IL-4, or TGF-β. There were four (11%) lines that did not produce any of the cytokines tested (Table 1, Group D). There was again no correlation between any particular pattern of cytokine expression and LCLs derived from a specific donor group. However, based on immunophenotyping, and cytokine and Ig production, the cell lines could be classified into four general categories (see Table 1, Groups A, B, C and D). Group A consists of 24 EBV-LCLs that are CD19+, CD20+, HLA-DR+, CD40+, CD23+, secrete very high (++++) levels of Ig and are strong cytokine producers. There was a strong correlation observed between CD23 expression and ++++ Ig secretion. The two cell lines in Group B differ in that although they exhibit strong cytokine production, including GM-CSF, they are CD23 −, secrete low levels of Ig and have fewer CD40 positive cells. Group C has seven cell lines that are HLA-DR+, CD23 − and secrete low levels of Ig, but have lost expression of CD19 and CD20 and produce low amounts of IL-10. Finally, Group D has four EBV-LCLs that express only HLA-DR and CD40, and produce no cytokines.

3.4. Comparison of high- and low-expressing EBV-LCLs

To verify that the EBV-transformed cell lines contain EBV viral genomic sequences, RT-PCR was performed on selected cell lines. Since BARTs are expressed in cultured lymphoblastoid cell lines (Chen et al., 1992; Brooks et al., 1993; Sadler and Raab-Traub, 1995), we analyzed three EBV cell lines from Group A (UKY-81-EBV, UKY-7663-EBV and UKY-CC1-EBV) and three from Group D (UKY-4712-EBV, UKY-78-EBV and UKY-85-EBV) for BART expression. We sought to compare cell lines expressing all (Group A) versus few (Group D) of the B cell markers we examined. As illustrated in Fig. 2, the expected 380-bp product was detected in each of the six cell lines tested, with UKY-81-EBV expressing the lowest amount. These data confirm the EBV-positive status of the cell lines following transformation.

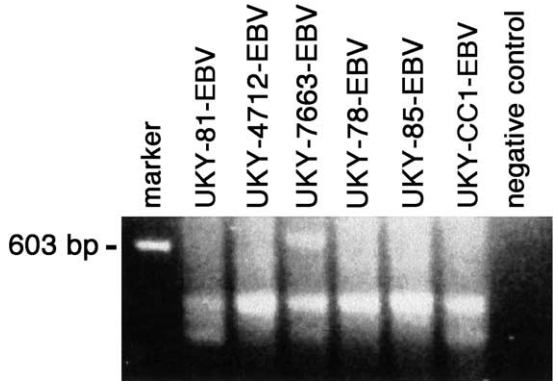

Fig. 2. RT-PCR analysis for *Bam*HI-A rightward transcripts. Ten micrograms of total cellular RNA was utilized for RT-PCR amplification of a 380-bp EBV-BART transcript. The samples tested were UKY-81-EBV (lane 2), UKY-4712-EBV (lane 3), UKY-7663-EBV (lane 4), UKY-78-EBV (lane 5), UKY-85-EBV (lane 6) and UKY-CC1-EBV (lane 7). Lane 8 is the negative control and the marker band in lane 1 is the 603-bp fragment of ϕX174 RF DNA digested with *Hae*III.

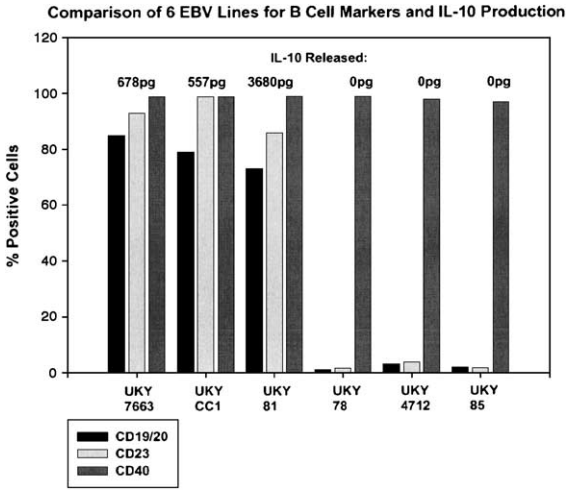

Fig. 3. Comparison of IL-10 positive and negative EBV-LCLs. Three EBV-LCLs from Group A (UKY-7663, UKY-CC1 and UKY-81) and three from Group D (UKY-78, UKY-4712 and UKY-85) in Table 1 are presented. Dark, light and medium shaded bars represent the B cell surface markers CD19/CD20, CD23 and CD40, respectively. IL-10 concentrations assessed by ELISA are listed above the bars for each cell line.

A comparison of B lineage cell surface markers and IL-10 production for these six EBV-LCLs is depicted in Fig. 3. All of these markers, CD19/CD20, CD23 and CD40, are expressed on a high percentage of cells from each of the Group A cell lines on the left. In contrast, the three Group D cell lines on the right only exhibit expression of CD40. Additionally, significant IL-10 production was detected in the Group A EBV-LCLs while Group D cell lines produced none.

4. Discussion

EBV-LCL immunophenotyping reports in the literature, together with the results in the present study, illustrate the variable expression of B cell surface markers on EBV-transformed cell lines. These observations most likely reflect the stage of differentiation of B cells at the time of the transformation event, although some contribution by the virus cannot be ruled out. While this panel of EBV-LCLs was strongly HLA-DR+, Hui et al. reported transformed B progenitors with a CD21+, CD20 −, HLA-DR − phenotype and more mature B cells as CD20+ and HLA-DR+. CD40, an important signaling molecule involved in B cell activation, was the only other marker detected in each of the EBV-transformed lines. The presence of these molecules suggests the EBV-LCLs devoid of CD19, CD20, CD23, κ or λ light chain expression are of B cell lineage. Moreover, the entire panel was negative for each T cell, NK cell, and monocyte and granulocyte cell surface marker examined.

An analysis of cytokine production by our EBV-LCL panel revealed that of the nine cytokines examined, IL-10, IL-6, IL-12p40 and TNF-α were detected in 89%, 49%, 51% and 32% of the EBV-LCLs, respectively (Table 1). IL-10 levels ranged from 13 pg/ml per 10^6 cells (UKY-84-EBV and UKY-80-EBV) to nearly 3.7 ng/ml per 10^6 cells (UKY-81-EBV) in 24 h. Additionally, IL-6 ranged from 6 pg/ml (UKY-99-EBV) to 843 pg/ml (UKY-97-EBV), IL-12p40 from 28 pg/ml (UKY-38-EBV) to 1.7 ng/ml (UKY-96-EBV) and TNF-α from 2 pg/ml (AML020299-EBV) to 62 pg/ml (UKY-CC1-EBV). These results lend support to the idea that IL-10 (Rochford et al., 1997; Beatty et al., 1997; Burdin et al., 1997) and IL-6 (Tosato et al., 1990; Yokoi et al., 1990; Tanner and Tosato, 1992) may serve as autocrine loops, since they are expressed in most of the lines. However, we identified four lines that produced neither cytokine, so these cytokines are not an absolute requirement for EBV-LCL growth. This observation is supported by a study that found that although 5/5 LCLs produced IL-10 and were negative for IL-4 and IL-6, there was no evidence that IL-10 provided an autocrine growth loop (Randhawa et al., 1997). We were unable to detect IL-12p70, IFN-γ, IL-4, or TGF-β produced by any of the lines, and GM-CSF was detected in a few supernatants. We did attempt to induce IL-12p70 production by cross-linking CD40 on three EBV-LCLs (UKY-96-EBV, UKY-97-EBV, and UKY-MEL1) that produce high levels of IL-12p40, but 24-h culture supernatants still tested negative. There are reports of variable expression of IL-12p40 (Rochford et al., 1997), IFN-γ and GM-CSF (Whittingham et al., 1993; Rochford et al., 1997), and no IL-4 expression (Rochford et al., 1997; Randhawa et al., 1997). Thus, it appears that EBV-LCLs may produce cytokines singly, in different combinations, or not at all. Moreover, together with the cell surface marker analysis, the only EBV-LCLs that produce significant amounts of cytokine(s) (Table 1, Groups A and B) are those with CD19 and CD20 expression.

It should be noted that the cell surface phenotype and cytokine production (or lack of) by each of the EBV-LCLs tested were stable over numerous passages in culture (data not shown). While data for cytokine production over 24 h are presented, 48, 72 and 96 h were also examined initially. As expected, an accumulation of cytokine(s) was observed only in the culture supernatants from EBV-LCLs secreting them after 24 h (data not shown). However, one potential variable in measuring cytokine production by cells in culture could be cytokine consumption. Future experiments could address this, for example, by testing to see whether blocking cell surface receptors for a given cytokine led to significantly increased amounts detected in the supernatants, or by RT-PCR analysis for the presence of mRNA transcripts for a given cytokine.

Immunoglobulin isotypes IgM and/or IgG were detected in culture supernatants from 24/37 (65%) of our cell lines. This is again most likely a result of the heterogeneity of the transformed B cell populations (Miyawaki et al., 1988). Finally, the presence of EBV genomic sequences was confirmed by RT-PCR analysis for EBV *Bam*HI-A rightward transcripts. The EBV-LCLs depicted in Fig. 2 were selected because three lines (UKY-81-EBV, UKY-7663-EBV and UKY-CC1-EBV) expressed all of the B cell markers and produced some cytokine(s) and immunoglobulin, and three (UKY-4712-EBV, UKY-78-EBV and UKY-85-EBV) were negative for everything except HLA-DR and CD40, and a low percentage of UKY-4712-EBV cells express λ light chains. These data verify that EBV genomic sequences are contained in each of these EBV-LCLs.

We observed a 100% correlation between CD23 expression and Ig secretion. This is not surprising, as both are associated with later stages of differentiation or an "activated" status, which corresponds to mature plasma cells (Kincade and Gimble, 1993). In addition, only those EBV-transformed cell lines expressing CD19 and CD20 produce significant cytokine(s) levels (see Table 1, Groups A and B). CD23 was present on 24/26 (92%) of these cytokine producing cell lines. Taken together, these data suggest that B cells transformed in later stages of differentiation are the ones to produce cytokine and Ig.

Our experience with in vitro expansions of NSCLC TIL and CTL has yielded variable results and led us to characterize our EBV-LCLs. The data presented clearly illustrate that all EBV-LCLs are not the same. Since IL-10 is an inhibitory cytokine for T cells, one should exercise caution when selecting an EBV-LCL for use as an allogeneic feeder cell line. We recommend utilizing an EBV-LCL that produces lower amounts or no IL-10 to ensure maximal T cell proliferation. Future experiments will compare the effectiveness of Group D EBV-LCLs as allogeneic feeders in in vitro T cell expansion cultures.

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