Infection of Human Endothelial Cells with Epstein-Barr Virus

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Summary

Interleukin-6 (IL-6) promotes growth and tumorigenicity of Epstein-Barr virus (EBV)-immortalized B cells, and is abnormally elevated in the serum of solid organ transplant recipients who develop EBV-positive posttransplant lymphoproliferative disease (PTLD), but not in control transplant recipients. Endothelial cells derived from PTLD lesions were found to secrete spontaneously high levels of IL-6 in vitro for up to 4 mo. We examined possible mechanisms for sustained IL-6 production by endothelial cells. Here, we show that EBV can infect endothelial cells in vitro. After 3–4 wk incubation with lethally irradiated EBV-positive, but not EBV-negative cell lines, a proportion of human umbilical cord–derived endothelial cells (HUVECs) expressed in situ the EBV-encoded small RNAs (EBER). Southern blot analysis after polymerase chain reaction showed EBV DNA in HUVEC that had been incubated with lethally irradiated EBV-positive cells, but not in the controls. Exposure of HUVECs to lethally irradiated EBV-positive but not EBV-negative cell lines induced IL-6 production that was sustained for up to 120 d of culture. These studies identify endothelial cells as targets for EBV infection and raise the possibility that this infection may be important in the life cycle and pathology of EBV.

EBV is a ubiquitous herpesvirus that has developed a successful strategy for infection, persistence, and spreading in humans (1). More than 90% of the population is chronically infected with EBV, almost always without symptoms. The key to the successful penetration of EBV in humans is infection and intermittent replication in the epithelium lining the oropharynx, events that ensure spread to new host, and establishment of lifelong latency, probably in the B cells (2).

Yet EBV infection is occasionally associated with disease (2). Lymphoproliferative disorders involving EBV-infected B cells arise frequently in the context of severe congenital or acquired immunodeficiency states (2). As many as 30% of solid organ transplant recipients may develop EBV-positive posttransplant lymphoproliferative disease (PTLD)[1] (3). The association of EBV-positive lymphoproliferative disease with severe immunodeficiency, combined with laboratory evidence supporting the existence of a variety of immune effector mechanisms potentially involved in control of EBV infections support the notion that immune surveillance is critical to limiting the proliferation potential of EBV-infected B cells (2). But other contributing factors may also be required for EBV to cause lymphoproliferative disease.

Recently, IL-6 was found to increase the tumorigenic potential of EBV-immortalized B cells in athymic mice (4, 5) and to serve as a growth factor for EBV-immortalized cells in vitro (6). IL-6 levels were also found to be elevated in serum or plasma of most solid organ transplant recipients with PTLD, but not in those with uncomplicated courses posttransplant, raising the possibility that IL-6 may play a role in the pathogenesis of PTLD (7). When incubated in vitro after separation into single cells, PTLD tissues were found to secrete high levels of IL-6 for up to 4 mo in culture (7). Cell separation experiments identified the endothelial cells as the principal source of IL-6 within PTLD tissue cultures.

The present study was undertaken to explore the mechanisms responsible for sustained IL-6 secretion by endothelial cells within PTLD lesions, and ultimately address the role of IL-6 in the pathogenesis of PTLD. We document here that primary endothelial cells are infectable with EBV in vitro and can produce high levels of IL-6 for up to 4 mo.

[1] *Abbreviations used in this paper:* EBER, Epstein-Barr virus–encoded small RNAs; HUVEC, human umbilical vein endothelial cells; LCL, lymphoblastoid cell lines; PTLD, posttransplant lymphoproliferative disease.

Materials and Methods

Cells, Cell Lines and Reagents. Primary cultures of human umbilical vein endothelial cells (HUVEC), obtained from the American Type Culture Collection (ATCC, Rockville, MD) were grown in RPMI 1640 medium (GIBCO BRL, Gaithersburg, MD), 15% heat-inactivated fetal bovine serum (FBS; Intergen Co., Purchase, NY), 20 U/ml porcine preservative-free heparin (Squibb-Marsam, Inc., Cherry Hill, NJ), and 100 μg/ml endothelial cell growth supplement (Calbiochem-Novabiochem Corp., La Jolla, CA). HUVEC stained positive (95–100% positivity) for von Willebrand factor and for CD31, as determined by indirect immunofluorescence using mouse mAbs to von Willebrand factor (Boehringer Mannheim Biochemicals, Indianapolis, IN) or to PECAM-1 (CD-31) (R & D Systems, Inc., Minneapolis, MN) followed by an affinity-purified, fluorescein-labeled goat anti–mouse reagent (Becton Dickinson Immunocytometry Systems, San Jose, CA). All 12 lymphoblastoid cell lines (VDS-O, DH, 3/22, To, Mary, RY, Rb, Rob, cb #2, TB, AVM, and IB4) were obtained by either spontaneous outgrowth of peripheral blood from EBV-seropositive individuals or by infection of B cells with the supernatant of the EBV producer marmoset cell line B95-8. The six EBV-negative Burkitt's lymphoma cell lines (BL-30, BL-41, ST48G, Ramos, JD38, and CA46) and the seven EBV-positive Burkitt's lymphoma cell lines (Ag876, Akata, P3HR-1, BL-30-B95, BL-41-B95, BL-41-P3HR-1), and B95-8 were kind gifts from Dr. I. T. Magrath (National Cancer Institute, National Institutes of Health, Bethesda, MD). All B cell lines were maintained in RPMI 1640 medium (GIBCO BRL) supplemented with 10% heat-inactivated FBS (Intergen Co.), 2 mM L-glutamine (GIBCO BRL), and 5 μg/ml gentamicin (Sigma Chemical Co., St. Louis, MO). All cell lines were mycoplasma free. Cells were irradiated in a ^{137}Cs γ irradiator at 1 Gy/min for 12 min (15,000 rad). Human rTNF-α was a gift from Genentech, Inc. (South San Francisco, CA); human rIL-6 was a gift from Sandoz Pharmaceutical (Basel, Switzerland); human rIL-10 was a gift from K. Moore (DNAX Research Institute, Palo Alto, CA); LPS from *Escherichia coli* 0127:B8 was purchased from Sigma; and a neutralizing purified goat antiserum to human TNF-α was purchased from R & D Systems. At a concentration of 1 μg/ml, this antiserum neutralized >90% of the biological activity due to 50 ng/ml human rTNF-α (Genentech).

Assays for IL-6, TNF, IFN-α, IL-4, IL-1β, and IL-10. The murine hybridoma cell line B9 (a gift from Dr. R. Nordan, Center for Biologics Evaluation and Research, Bethesda, MD) was used in a standard assay for IL-6 bioactivity. 1 U of IL-6 bioactivity in this assay is defined as the activity inducing half-maximal proliferation of B9 cells. An IL-6 concentration of 1 U/ml corresponds to ~20 pg of *E. coli*–derived human IL-6 used throughout as a laboratory standard (a gift from Sandoz Pharmaceuticals). The fibroblast murine cell line L921 (a gift from Dr. T. Gerrard, Center for Biologics Evaluation and Research) was used in a standard assay for TNF bioactivity. 1 U of TNF bioactivity in this assay is defined as the activity inducing one-half maximal lysis of L921 targets. A TNF concentration of 1 U/ml corresponds to ~100 pg of a human rTNF-α preparation (a gift from Genentech, Inc.). Human IL-4 was measured in a biological assay based on its capacity to induce CD23 expression on the Burkitt's lymphoma cell line, Ramos, as described (8). IL-4 concentrations were determined by comparison with a laboratory standard IL-4 preparation (Immunex Co., Seattle, WA). The sensitivity of this assay was ~50 pg/ml. IFN-γ levels were determined by ELISA, essentially as described (8). IFN-γ activity was determined by comparison with a standard IFN-γ preparation (Genentech, Inc.). The sensitivity of this assay was ~10 pg/ml. IL-1β was measured by a standard ELISA (R & D Systems) by a comparison with a standard IL-1β preparation; the sensitivity of this assay was ~15 pg/ml. Human and/or viral IL-10 was measured by a specific ELISA (Biosource International, Camarillo, CA). This ELISA uses a murine anti–IL-10 (human and viral) mAb (IgG1 kappa clone B-S10) to coat the microwells, as well as a biotinylated rabbit anti–IL-10 (human and viral) polyclonal antibody to capture bound IL-10. Recombinant (*E. coli*–derived) purified human IL-10 (Biosource International) was used as the standard. The lowest limit of sensitivity of the assay was determined to correspond to 11.7 pg/ml purified human IL-10 standard and 3.1×10^{-3} U/ml viral IL-10 (supernatant of COS cells transiently transfected with the EBV BCRF-1 (viral IL-10) gene, a gift from K. Moore, DNAX Research Institute). 1 U of viral IL-10 is defined as the amount inducing half-maximal inhibition of IFN-γ production in mouse T cell clones (9).

In Situ EBV Hybridization. The procedure was performed essentially as described (10). Modification of the technique included a 10% formalin fixation to permit the use of culture chamber slides (Nunc, Inc., Naperville, IL) from which the upper structure had been removed. The cell-adherent slides were rehydrated in serial graded ethanol washes (70% and 50%) and subjected to pronase E (10 μg/ml; Sigma) digestion for 7 min at 37°C. Slides were then exposed to a sense and antisense probe to the EBV-encoded small RNA EBER1 in a 50% formamide buffer for hybridization in a volume sufficient to fully cover the slide. Slides were covered with parafilm during the 2-h hybridization period at 55°C. Hybridization was followed by a series of stringent washes at room temperature with 2× SSPE (NaCl, NaH_2PO_4, EDTA solution)/0.1% SDS for 5 min, at 55°C with 0.1% SSPE/0.1% SDS for 5 min, at room temperature with 2× SSPE for 1 min, and then incubated for 1 h at room temperature with an alkaline phosphatase–conjugated goat antibody to digoxigenin (Genius 3 kit; Boehringer Mannheim) diluted 1:500 into 150 mM NaCl/100 mM Tris solution containing 1% goat serum. Slides were washed in 150 mM NaCl/100 mM Tris for 5 min, and in 100 mM NaCl/50 mM $MgCl_2$ for 5 min, before development in a 5-bromo-4 chloro-3-indolyl phosphatase (NBT)-based color reaction. Slides were counterstained with eosin, dehydrated in serial graded ethanol washes and xylene, and mounted. For the preparation of the probes, plasmids with the EBER1 coding sequences and T7 RNA polymerase promoters appropriately positioned to produce sense or antisense transcripts in vitro were provided by Dr. R. Ambinder (Johns Hopkins University, Baltimore, MD). Plasmids were linearized and transcribed in vitro for 2 h at 37°C in the presence of digoxigenin-UTP in the appropriate transcription buffer (Genious 3 kit; Boehringer Mannheim). After treatment with DNAse I at 37°C for 15 min, the probes were phenol-chloroform extracted and ethanol precipitated. Before use, the activity of the probes was determined using a reference standard (Genius 3 kit; Boehringer Mannheim).

PCR and Southern Blotting. To detect EBV DNA, we used a PCR strategy based on that of Riddler et al. (11). Aliquots of DNA were extracted from 10^5 cells or from pellets derived from centrifugation of culture supernatants and were subjected to a PCR protocol designed to amplify a 269-bp segment within a highly conserved single-copy region of *EBNA1*. Amplifications were carried out in 100 μl containing 50 mM KCl, 10 mM Tris-HCl pH 9.0, 0.1% Triton X-100, 1.5 mM $MgCl_2$, 200 mM of each dNTP, 1 μM of each primer (GTCATCATCATCCGGGTCTC and TTCGGGTTGGAACCTCCTTG), and 2.5 U of Taq DNA polymerase (Promega Biotech Corp., Madison, WI)

for 35 cycles of 1 min 95°C, 2 min 63°C, and 1 min 73°C. The amplification products were detected by Southern blotting. The probe was a 215-bp segment of DNA internal to the above primers and was generated by PCR amplification from a plasmid containing the BamHI K fragment of the EBV genome of B95.8 (11) using the primers CCTCCAGGTAGAAGGCCATT and ACCACGATGCTTTCCAAACC. The membrane was exposed to XAR film (Eastman Kodak Co., Rochester, NY) overnight at −70°C with an intensifying screen.

Cord Blood Immortalization. HUVEC supernatants were tested for the presence of infectious EBV by a cord blood immortalization assay. Cord blood mononuclear cells obtained from heparinized cord blood were seeded in flat-bottom microtiter wells (Costar Corp., Cambridge, MA) at 2×10^5 cells in 0.1 ml culture medium (RPMI 1640 [GIBCO BRL] supplemented with 10% heat-inactivated FCS [Intergen Co.], 2 mM L-glutamine [GIBCO BRL] and 5 μg/ml gentamicin [Sigma Chemical Co.]). Culture medium, supernatant of the B95-8 cell line, or test supernatants were added (0.1-ml aliquots) in triplicate to cord blood mononuclear cells, and the cultures were incubated for 3–4 wk. A microculture was considered morphologically immortalized when large single cells and clumps of large cells were recognized on phase-contrast microscopy. Selected cultures with microscopic evidence of outgrowth were harvested along with control cultures and the cells were washed; DNA was extracted from cell pellets and subjected to PCR amplification of a 269-bp segment within EBNA1, followed by Southern blotting as described above. A cord blood culture was considered immortalized by EBV if it appeared morphologically immortalized and contained EBNA1 sequences.

Results

IL-6 Production by Endothelial Cells Exposed to EBV-immortalized B Cells. Previous experiments demonstrated that endothelial cells derived from lymphoproliferative lesions of patients with PTLD produce high levels of IL-6 in culture for periods of weeks (7). With the goal of establishing the mechanism of IL-6 induction in these cells, we tested whether normal human endothelial cells are stimulated to secrete IL-6 by EBV-immortalized cells. To this end, HUVECs were cultured in 24-well plates until confluency. Subsequently, the effects of four EBV-immortalized cell lines were tested by adding 10^6 irradiated (15,000 rad) cells to each HUVEC-containing well or to empty wells. Cultures were then continued with periodic (once or twice per week) exchange of one-half (1 ml) culture medium with fresh medium. As shown in Fig. 1, HUVEC culture supernatants incubated alone contained at most 218 IL-6 U/ml during a 66-d culture period. In contrast, culture supernatants of HUVEC incubated in parallel with irradiated EBV-immortalized cells from four separate cell lines contained much higher IL-6 levels, reaching 4,790 and 28,840 U/ml during the same incubation period. Because culture supernatants of the heavily irradiated EBV-immortalized cell lines incubated alone contained at most 269 IL-6 U/ml, and because the cell viability of EBV-immortalized cells 11 d after irradiation was <2% both when cultured alone and on HUVEC monolayers, we concluded that HUVECs were the likely source of IL-6 in the cultures.

Regular inspection of HUVEC cultures by phase-contrast microscopy at intervals during incubation either alone or with irradiated EBV-immortalized cells showed a progressive loss of confluency in the cell monolayer, presumably secondary to cell death and subsequent detachment from plastic. Initially, HUVECs constituted a homogeneous population of closely opposed, large, polygonal cells with a centrally located nucleus and indistinct cell borders. By day 30 or 40 of culture, only sparse adherent cells were visible, but these cells maintained the original aspect of large, polygonal cells with centrally located nucleus.

To test further IL-6 production in this setting, we repeated the study to include 12 lymphoblastoid cell lines (LCL) obtained by either spontaneous or EBV (B95-8 strain)–induced immortalization; six EBV-negative Burkitt's lymphoma cell lines; six EBV-positive Burkitt's lymphoma cell lines (including three EBV-converted Burkitt's lymphoma cell lines BL-30[B95-8] and BL-41 [B95-8 and P3HR-1], and three EBV-producer cell lines P3HR-1, Ag876, and Akata); and the EBV-producer marmoset B95-8

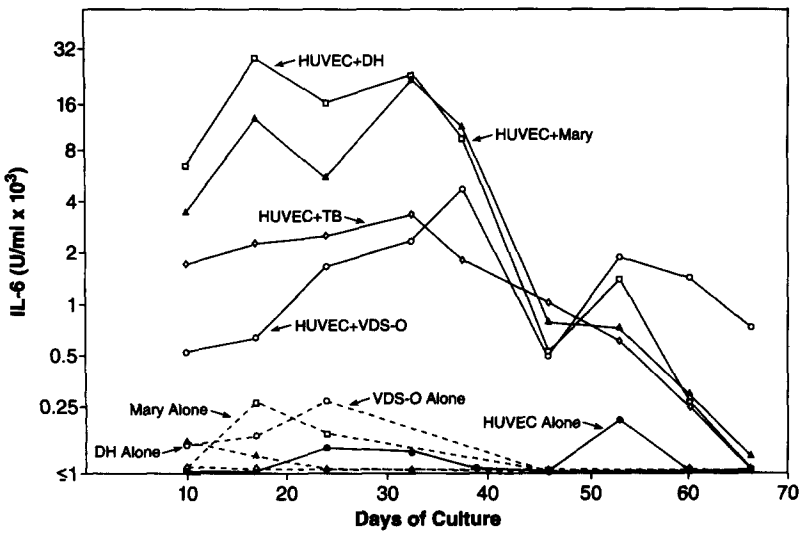

Figure 1. Levels of IL-6 in cultures of endothelial cells incubated with EBV-immortalized cell lines. HUVEC were first cultured to confluency in 24-well plates and then incubated with 10^6 irradiated (15,000 rad) EBV-immortalized cells from the cell lines DH, Mary, TB and VDS-O. Controls included HUVEC and irradiated B cell lines cultured alone. Cultures were periodically fed as needed, and supernatants were tested for IL-6 content at the indicated time points.

cell line (Fig. 2). Cell cultures were maintained for 28–112 d, and culture supernatants were tested for IL-6 at 3–10 d intervals throughout the culture period. We looked for the highest IL-6 levels achieved in the supernatants (Fig. 2). The mean highest IL-6 level measured in supernatants of HUVECs cultured alone was 118 ×/: 1.2 U/ml, a level significantly lower than that measured in the supernatants of HUVECs cultured with irradiated LCL (4,574 ×/: 1.5) and in the supernatants of HUVECs cultured with the irradiated EBV-positive lymphoma cell lines (1,572 ×/: 1.9), as determined by group comparison using Dunnett's test. In contrast, the mean highest IL-6 level in supernatants of HUVECs alone was not significantly different (Dunnett's test) from that measured in supernatants of HUVECs cultured with irradiated EBV-negative lymphoma cell lines (184 ×/: 1.2). As expected, culture supernatants of all irradiated cell lines incubated in the absence of HUVEC contained little IL-6. These experiments demonstrated that HUVECs can be induced to secrete IL-6 by a variety of EBV-positive but not EBV-negative cell lines.

Kinetics of IL-6 production by endothelial cells exposed to LCL or to EBV-producing cell lines (Ag876, B95-8, Akata, and P3HR-1) showed that peak cytokine levels were achieved, on the average, on day 24 (range 9–85) of culture. IL-6 levels, however, were found to be above controls (endothelial cells alone and irradiated LCL alone), on the average, up to day 47 (range 19–93) of culture.

Mechanisms of IL-6 Production by Endothelial Cells Exposed to EBV-infected Cell Lines. Previous studies have shown that endothelial cells can be induced to produce IL-6 by a variety of signals, including LPS, IL-1β, TNF-α, IL-4 alone or with IFN-α, and to a limited extent, IL-10 (12–14). It was unlikely that LPS was responsible for IL-6 induction in our system because the endotoxin level in the culture supernatants of four IL-6 inducing B cell lines was <0.06 endotoxin U/ml, a dose not different from that of medium alone. It was also unlikely that IL-1β, IL-4, and IFN-γ were involved in our system because IL-1β, IL-4, and IFN-γ were undetectable in the culture supernatants of eight of eight LCL and in four of four of the EBV-positive Burkitt's lymphoma lines tested (not shown) using ELISAs with detection limits 10–20-fold lower than the amounts of cytokine required for induction of IL-6 in endothelial cells. LCLs, however, are known to secrete TNF (15) and IL-10 (16), and we determined that 2-d culture supernatants of eight irradiated LCL (10^6 cells per ml) contained a mean of 38.8 TNF U/ml, and six of eight had detectable IL-10. Addition of TNF-α (1.5–50 ng/ml, corresponding to 4–135 U/ml) to HUVEC resulted in the induction of IL-6 (not shown). The kinetics of IL-6 production induced by TNF-α, however, differed from those induced by LCL in that peak IL-6 levels were measured, on the average, on day 9 with TNF-α at the highest dose (earlier at lower doses) versus day 24 with EBV-positive lines. In addition, the IL-6 response to TNF was undetectable, on the average, by day 38 (or earlier at lower doses), but continued up to day 47, on the average, with exposure to the cell lines (not shown). Measurements of IL-6 and TNF levels in HUVEC supernatants exposed to the Ag876 lines during a 126-d period revealed a discordance between TNF, which was detectable only initially in the culture, and IL-6, which was detectable throughout (Fig. 3 *A*). Also, addition of a neutralizing antibody to human TNF-α had minimal effects on IL-6 production by LCL-exposed HUVEC after a modest diminution early in the culture (Fig. 3 *B*). In control cultures, the anti–TNF-α antibody markedly reduced IL-6 production induced by 10 ng/ml TNF-α (Fig. 3 *B*). In related experiments, we found that human rIL-10 (10 ng/ml) did not increase IL-6 production by HUVECs (not shown). The combined effects of TNF-α (ng/ml) plus IL-10 (10 ng/ml) on IL-6 production by HUVECs was similar to that of TNF-α (10 ng/ml) alone. Finally, in three sepa-

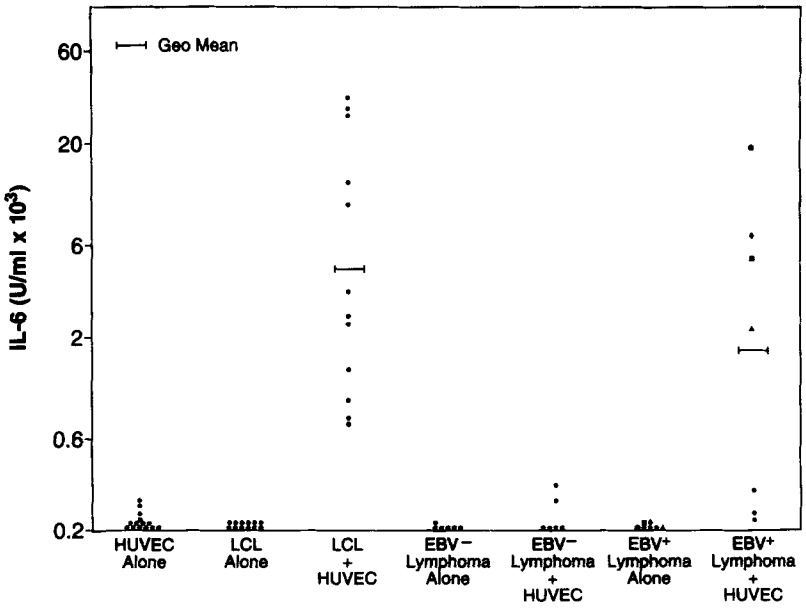

Figure 2. IL-6 production in endothelial cell cultures incubated with EBV-positive and EBV-negative B cell lines. The culture conditions are identical to those described in the legend to Fig. 1; time in culture varied from 28 to 112 d. The results reflect the highest IL-6 level measured in the culture supernatants tested at 3–10-d intervals.

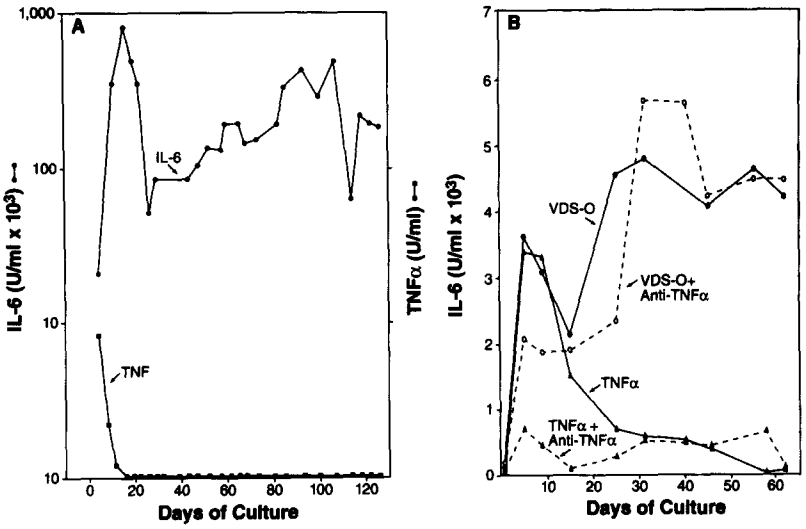

Figure 3. TNF production by EBV-infected cells is not solely responsible for IL-6 production in cultures of HUVEC with EBV-infected cells. (*A*) Levels of IL-6 and TNF in supernatants of endothelial cells incubated with the EBV-positive Ag876 cell line. The culture conditions are identical to those described in the legend to Fig. 1. The results reflect IL-6 and TNF levels measured in supernatants obtained at the indicated culture time points. (*B*) IL-6 production by endothelial cells exposed to TNF-α alone, TNF-α plus anti–TNF-α–neutralizing antibody, irradiated EBV-positive VDS-O cells alone, or VDS-O cells plus anti–TNF-α–neutralizing antibody. Confluent HUVEC cultures received either TNF-α (10 ng/ml) alone or with a neutralizing antibody to TNF-α (1 μg/ml), or irradiated (15,000 rad) VDS-O cells (10^6 per well) alone or with a neutralizing antibody to TNF-α (1 μg/ml). The results reflect IL-6 levels in the culture supernatants at various time points.

rate experiments (not shown), supernatants of two EBV-immortalized cell lines (VDS-O and DH), of three EBV-positive Burkitt's lymphoma cell lines (Ag876, Akata, and P3HR-1), and of the EBV-producer B95-8 cell line added once to HUVEC cultures (1:2 dilution) failed to induce IL-6 production in these cells during a 20-55 d period. These findings suggested that any cytokines that might be released by EBV-immortalized cells cannot account for IL-6 secretion by HUVECs exposed to EBV-positive cell lines.

Endothelial Cells Are Infected by EBV and Replicate Infectious EBV. The observation that HUVECs exposed to EBV-infected irradiated cell lines produce higher levels of IL-6 than HUVECs cultured alone for a prolonged period, up to 125 d, raised the possibility that the endothelial cells might become infected with EBV, and that EBV infection might promote IL-6 secretion. To address this possibility, HUVECs were first cultured for 3–4 wk in chamber slides either alone or with irradiated B cell lines (10^5 per w), and were then examined for evidence of EBV infection. After the removal of all cells and cell fragments in suspension, glass-adherent cells were fixed and subjected to in situ hybridization using probes for the highly transcribed EBV-encoded small RNAs (EBERs). In situ hybridization with probes in the sense orientation was used as a control. Using this technique, we found that HUVECs exposed to irradiated EBV-positive lymphoblastoid cell lines (DH, VDS-O, RY, and TB) or to the EBV-positive Burkitt's Ag876 cell line expressed EBV-specific EBER1 mRNA. Control HUVECs either cultured alone or exposed to EBV-negative Burkitt's cells (PCA46 and JD38) were EBV negative by the same assay. In one experiment, >50% of the adherent cells exposed to VDS-O cells were found to be EBV-positive (Fig. 4 *A*). In most other experiments, however, the level of EBV positivity was in the 5–10% range. It should be noted, however, that in most cases the percent of cell positivity could not be accurately determined because a proportion of the adherent cells was lost during fixation and staining. The cells staining positive for EBER were likely to be HUVECs and not the EBV-positive B cells because of their elongated morphology and adherence to glass. Indeed, control chamber slides containing the irradiated EBV-positive cells alone (no endothelial cells) had no visible glass-adherent cells. These findings suggested that upon culture with irradiated EBV-positive cell lines, HUVECs become infected with EBV.

To confirm this finding, we looked for the presence of the EBV-DNA in HUVEC through PCR amplification of the *EBNA1* gene followed by Southern blotting of the PCR products. Monolayers of HUVECs were cultured either alone or with irradiated EBV-infected cells from the lymphoblastoid cell line VDS-O (10^6/ml). Irradiated VDS-O cells were also cultured alone (2×10^6/ml). After 9, 16, 24, and 30 d incubation, 10^5 live adherent cells were obtained from HUVEC cultures. At the same time points, nonadherent cells and cell fragments were recovered from cultures of HUVEC incubated with irradiated VDS-O cells, and of irradiated VDS-O cells cultured alone. Because the viabilities of the nonadherent cell populations recovered from cultures of irradiated VDS-O cells alone were 9–12, 0–1, 0, and 0% after 9, 14, 24, and 30 d incubation, but it was possible that EBV DNA might still be present, we subjected to PCR amplification all nonadherent cells and cell fragments recovered from centrifugation of 3-ml culture supernatants from the cocultures (HUVEC plus irradiated VDS-O cells) and from cultures of irradiated VDS-O cells cultured alone. The results of Southern blotting after PCR amplification (Fig. 5) show that HUVEC cultured alone (lanes *a*) were negative for *EBNA1* at all time points (days 9, 16, 23, and 30), but HUVECs exposed to irradiated VDS-O cells (lanes *b*) were positive for *EBNA1* at all time points. In contrast, the irradiated (15,000 rad) EBV-positive VDS-O cells (lanes *d*) scored positively for *EBNA1* only at the first time point, day 9 after irradiation, but they scored negatively for *EBNA1* at all later time points (16, 23, and 30 d after irradiation). The nonadherent cell population recovered from cocultures of HUVEC and irradiated VDS-O cells (lanes *c*) scored positive for *EBNA1* at all time points (days 9, 16, 23, and 30). Because the irradiated VDS-O cells cultured alone scored negatively for *EBNA1* on 16 d after irradiation and thereafter, we concluded that

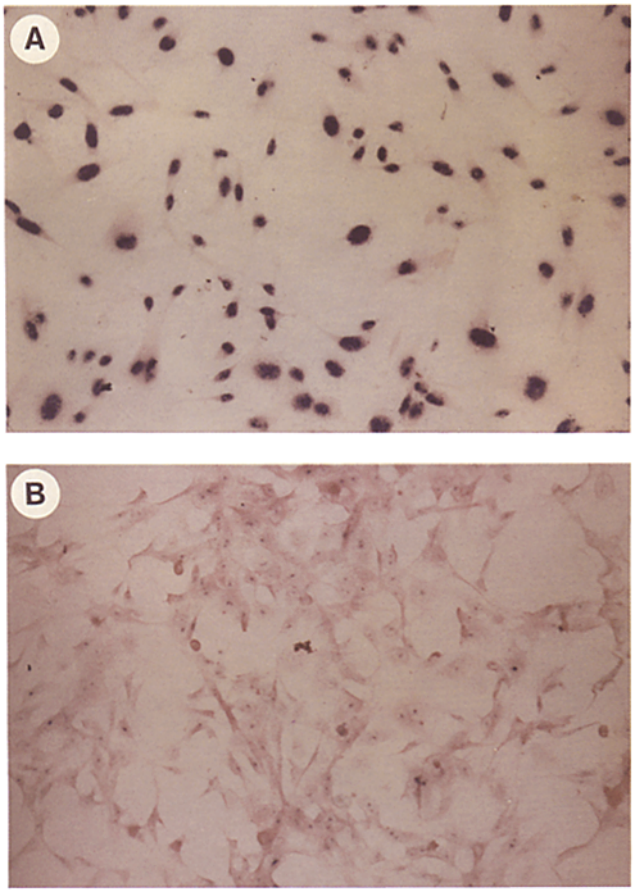

Figure 4. Detection of EBER1 transcripts in endothelial cells exposed to irradiated EBV-immortalized cells. In situ hybridization with digoxigenin-labeled EBER-specific antisense probes showing intense EBER positivity in cell nuclei (*A*). Control hybridization with EBER-specific sense probes (*B*). Slides were counterstained with eosin. ×800.

the *EBNA1* positivity of this nonadherent cell population (derived from the coculture of HUVECs plus irradiated VDS-O cells) beyond day 9 of culture had resulted from EBNA-positive HUVECs that had detached from the plastic surface.

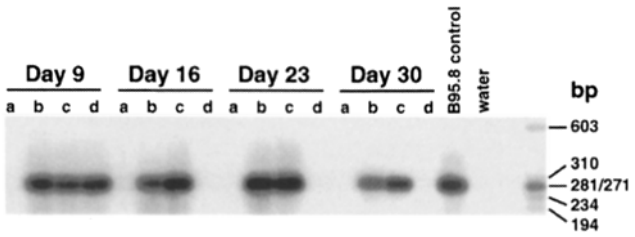

Figure 5. Detection of EBV-DNA in endothelial cells cultured with irradiated EBV-immortalized cells. Confluent HUVEC cells were either cultured alone or with irradiated (15,000) lymphoblastoid cells (VDS-O line 10^6 cells per well). Irradiated VDS-O cells were also cultured alone. At the indicated time points (9, 16, 23, and 30 d), HUVEC cultured alone (*a*), adherent cells from cultures of HUVEC plus irradiated VDS-O cells (*b*), nonadherent cells from HUVEC cultures plus irradiated VDS-O cells (*c*), and irradiated VDS-O cells cultured alone (*d*) were harvested and subjected simultaneously to PCR amplification of the *EBNA1* region with appropriate primers. The results reflect Southern blot hybridization with an *EBNA1*-specific probe.

In additional experiments, we examined whether EBV-infected HUVECs replicate infectious EBV capable of immortalizing cord blood lymphocytes. To this end, aliquots of culture supernatants from HUVECs incubated either in medium alone or with irradiated (15,000 rad) EBV-positive lymphoblastoid cells (VDS-O cell line) were harvested on days 8 or 16 of culture, and 0.1 ml was transferred in triplicate to microtiter cultures containing cord blood mononuclear cells (2×10^5 in 0.1 ml culture medium). Supernatants of the EBV-producer B95-8 cell line and complete HUVEC culture medium served as controls. A cord blood microculture was considered morphologically immortalized when both large single cells and clumps of large cells were recognized on phase-contrast microscopy. As shown in Table 1, a proportion of cord blood mononuclear cell cultures developed evidence of immortalization after exposure to supernatants of HUVEC cultured for 8 or 16 d with irradiated EBV-immortalized cells. In contrast, there was no evidence of immortalization in cord blood mononuclear cell cultures exposed to supernatants of HUVEC cultured

Table 1. *Detection of Infectious EBV in Culture Supernatants of HUVEC*

Test samples*	Immortalized cultures‡ (No. of positive wells/ total no. of wells) Experiment 1	Experiment 2
HUVEC medium	0/3	0/3
RPMI medium	0/3	0/3
B95-8 supernatant	3/3	3/3
8-d supernatant irradiated VDS-O	0/9§	ND
16-d supernatant irradiated VDS-O	ND	0/6§
8-d supernatant HUVEC	0/9	ND
16-d supernatant HUVEC	ND	0/6
8-d supernatant HUVEC + irradiated VDS-O	10/12‖	ND
16-d supernatant HUVEC + irradiated VDS-O	ND	2/6¶

*An aliquot (0.1 ml) of the indicated test samples was added to microtiter wells containing freshly isolated cord blood mononuclear cells (2×10^5 in 0.1 ml culture medium), and the cultures were incubated for 3–4 wk.

‡A cord blood culture was considered immortalized if it contained large single cells and clumps of large cells after 3–4 wk of culture, as determined by phase-contrast microscopy.

§A pool of four (out of nine) wells and a pool of all six wells tested negative for *EBNA1* DNA.

‖3 of 10 immortalized wells tested individually were positive for *EBNA1* DNA.

¶A pool of the two immortalized wells tested positive for *EBNA1* DNA.

ND, not done.

alone or to supernatants of EBV-immortalized VDS-O cells irradiated 8 or 16 d earlier and then incubated. The presence of EBV DNA in morphologically immortalized cord blood cultures was assessed through PCR amplification of *EBNA1*-specific sequences followed by Southern blotting. Three individual wells and a pool of two wells scored positively for *EBNA1*. In contrast, pools of wells containing cord blood mononuclear cells exposed to supernatants of EBV-immortalized cells irradiated 8 or 16 d earlier did not appear immortalized, and they tested negative for *EBNA1*.

Together, these findings demonstrate that HUVECs exposed to lethally irradiated EBV-immortalized cells become EBV-infected, and they can replicate infectious EBV.

Discussion

In the present studies, we have demonstrated that after incubation with lethally irradiated EBV-infected cells, human umbilical cord–derived endothelial cells become infected with EBV and secrete high levels of IL-6 for extended periods of culture. Although it is known that endothelial cells can be induced to secrete IL-6 by a variety of signals (12, 14), it has not been previously reported that endothelial cells can be infected with EBV. The evidence supporting the assertion that endothelial cells can be infected with EBV derives from in situ hybridization studies that have detected the presence of EBERs, as well as from Southern analyses after PCR amplification that have demonstrated the presence of *EBNA1* sequences in the endothelial cells. The EBV specificity of each of these assays strongly supports the notion that endothelial cells can be infected with EBV in vitro.

The relationship between EBV infection and IL-6 secretion in endothelial cells was not fully elucidated here. It is likely, however, that EBV infection directly or indirectly stimulates IL-6 production by endothelial cells in the present system. It would be difficult to envision an alternative mechanism by which endothelial cells might sustain increased IL-6 production for a period longer than 120 d in response to irradiated cells that are all dead 7–9 d after 15,000 rad irradiation. Furthermore, incubation of endothelial cells with a number of EBV-negative irradiated B cell lines was not associated with increased IL-6 production, further suggesting a link between EBV and the IL-6-inducing signal. It is interesting to note that in six separate attempts we have failed to infect HUVECs with EBV by exposing them to culture supernatants of the B95-8 and Ag876 cell lines that were found to contain $\sim 10^3$ infectious units per ml (results not shown). It remains to be established whether this resulted from insufficient virus or from a requirement for signals other than the virus itself for infection of endothelial cells.

The nature of EBV infection in endothelial cells was not fully elucidated here. Two preliminary observations were made, however (results not shown). In almost all experiments, endothelial cells exposed to EBV-immortalized cells and producing high levels of IL-6 lived longer than endothelial cells cultured in medium alone or with EBV-negative cell lines. This suggested that EBV infection might provide a survival advantage to endothelial cells. Also, in two experiments, we documented the presence of immortalizing EBV in the culture supernatant of endothelial cells that had been exposed to irradiated EBV-immortalized cells 2 wk earlier, but not in control supernatants of HUVEC cultured alone or in supernatants of EBV-immortalized cells that had been irradiated 8 or 16 d earlier, indicating that EBV replication had occurred in the endothelial cells. Another issue that remains unclear is how EBV enters HUVECs. Endothelial cells of small vessels were reported to stain positive for the EBV receptor molecule CD21 (17, 18), but using immunofluorescence, we found no evidence for the presence of CD21 on HUVECs (not shown). The presence of EBV receptors on epithelial cells and on malignant T cells has been difficult to document, even though these cell types are EBV infectable (19–21). The observation that supernatants of EBV-producing and EBV-immortalized cell lines failed to induce EBV infection and IL-6 production in HUVECs suggests that cell-to-cell contact may render EBV infection more efficient at low virus titers. Future studies will address both EBV gene expression in endothelial cells and EBV entry.

The observation that human endothelial cells are infectable with EBV and can replicate infectious EBV in vitro raises the possibility that these cells might also become infected with EBV in vivo. We have looked for EBV in the endothelial cells of two biopsy specimens of PTLD tissues by in situ hybridization and found them to be EBV negative. Because only a small fraction of endothelial cells within PTLD lesions might be EBV-infected, however, a more extensive and rigorous search will be needed to fully evaluate their EBV status. On the basis of the in vitro results reported here, we would predict that prolonged contact of EBV-producing cells with endothelial cells will result in EBV infection of the endothelial cells. EBV replication is believed to occur in some PTLD lesions (22), and the possibility of prolonged contact of EBV producing cells with endothelial cells within PTLD lesions exists. As noted here, IL-6 secretion is likely to be one of the outcomes of infection with EBV in vitro. Because IL-6 is known to represent a growth factor for EBV-infected B cells (6, 23), endothelial cell infection with EBV and the associated IL-6 production may constitute a local stimulus for PTLD progression.

Could endothelial cells represent a site of EBV infection in the normal life cycle of the virus, either during primary or latent infection of otherwise healthy individuals? There is no direct evidence that cell types other than epithelial cells and B cells are normally infected with EBV in vivo (1, 2). There are several missing or uncertain links in the life cycle of EBV in this host, however, particularly the relationship that exists between EBV-productive infection in the differentiating epithelium of the oropharynx and EBV latency in the B cell compartment (2). We know that EBV is periodically replicated in the oropharynx throughout life,

and although many EBV variants are generated during viral replication, the same EBV isolate is detected in the saliva of an infected individual over a period of many years (24). It was proposed that endogenous virus must be fed to the oropharyngeal epithelium from a latent reservoir where the virus is protected from change associated with extensive replication (25). But the only other known site of EBV persistence, the B cells, is an unlikely source of virus because of the latent nature of EBV infection here (1). Could endothelial cells serve such a role?

EBV-seropositive normal individuals harbor a small and relatively constant number of EBV-infected B cells in the peripheral blood (2, 26) despite EBV-specific cytotoxic T cell precursors that account for 1 per 10^3–10^4 circulating T cells (25, 27). How can EBV-infected B cells escape T cell killing? Since the specificity of the viral-specific cytotoxic cell population was mapped to a number of viral latency genes, including *EBNA4*, *EBNA2*, *EBNA3a*, *EBNA3c*, and *LMP1* (28–33), but not to *EBNA1*, it was proposed that EBV-infected B cells in seropositive normals may express *EBNA1* only or mutated viral epitopes selected for CTL resistance (25, 34). But recently, several studies have documented expression of *EBNA4* and other EBV latency genes in the circulating B cells (35, 36), suggesting that at least some virally infected B cells are targets of T cell cytotoxicity. Could endothelial cells in EBV-seropositive normals represent a reservoir for EBV that continuously infects B cells, replacing those destroyed by cytotoxic T cells?

In addition to B lymphocytes and epithelial cells, T lymphocytes from certain lymphoproliferative diseases were found to harbor EBV (20, 21). Reed-Sternberg and Hodgkin's cells are known to carry EBV genomes in ~50% of Hodgkin's lymphoma specimens (10, 37). Furthermore, some follicular dendritic cells were successfully infected with EBV in vitro (38). Recently, smooth muscle tumors in either immunosuppressed AIDS patients or recipients of liver transplant were found to be EBV infected (39, 40).

Many questions remain to be addressed, including the nature of endothelial cell infection with EBV, the pattern of gene expression by the endothelial cells, and the relevance of in vitro endothelial cell infection with EBV to the in vivo situation, both in diseased and normal individuals. The novel observation that endothelial cells are infectable by EBV, however, has the potential to clarify some critical issues of EBV infection that remain unresolved.

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