Persistent Productive Epstein-Barr Virus Replication in Normal Epithelial Cells In Vivo

Dennis M. Walling,1 Catherine M. Flaitz,3 C. Mark Nichols,2 S. David Hudnall,2 Dennis M. Walling,1 Catherine M. Flaitz,3 and Karen Adler-Storthz4

Productive Epstein-Barr virus (EBV) replication characterizes hairy leukoplakia, an oral epithelial lesion typically occurring in individuals infected with human immunodeficiency virus (HIV). Serial tongue biopsy specimens were obtained from HIV-infected subjects before, during, and after valacyclovir treatment. EBV replication was detected by Southern hybridization to linear terminal EBV genome fragments, reverse-transcriptase polymerase chain reaction amplification of EBV replicative gene transcripts, immunohistochemical detection of EBV replicative protein, and in situ hybridization to EBV DNA. EBV replication was detected in both hairy leukoplakia and normal tongue tissues. Valacyclovir treatment completely abrogated EBV replication in vivo, resulting in resolution of hairy leukoplakia when it was present. EBV replication returned in normal tongue epithelial cells after valacyclovir treatment. These data suggest that normal oral epithelium supports persistent EBV infection in individuals infected with HIV and that productive EBV replication is necessary but not sufficient for the pathogenesis of oral hairy leukoplakia.

The Epstein-Barr virus (EBV) is a human herpesvirus that persistently infects up to 95% of adults worldwide. EBV infection is associated with important human diseases, including infectious mononucleosis syndrome, malignant lymphomas, and nasopharyngeal carcinoma.

EBV infection and persistence are incompletely understood. Latently infected circulating B lymphocytes are believed to be one site of life-long EBV persistence [1]. Yet, EBV must productively replicate at mucosal surfaces to transmit infection to new hosts. Early studies suggested that EBV productively replicates in oral epithelial cells [2–4], but oral epithelial sites of replication have not been identified. More recent data suggest that EBV reactivates and productively replicates directly within B lymphocytes trafficking through oral mucosal lymphoid tissues [5–8]. Thus, the role of oral epithelial cells in the persistence and transmission of EBV infection remains uncertain.

Hairy leukoplakia is a common, nonmalignant oral disease of individuals infected with human immunodeficiency virus (HIV) [9]. Hairy leukoplakia typically occurs on the lateral borders of the tongue and is uniquely characterized by abundant productive EBV replication within histologically abnormal epithelial cells [10]. Inhibition of productive EBV replication by antiviral agents results in the resolution of hairy leukoplakia, which suggests that EBV replication is important in the pathogenesis of the lesion [11]. In this study, we examined the role of productive EBV replication in the pathogenesis of hairy leukoplakia and the role of oral epithelial cells in the persistence of EBV infection in HIV-infected individuals.

Materials and Methods

EBV treatment and collection of human tissues. Two HIV-seropositive subjects were treated orally with 1000 mg of valacyclovir (Valtrex; GlaxoSmithKline) every 8 h for 28 days, to inhibit productive EBV replication. Three serial lateral tongue epithelium biopsy specimens were obtained from each subject immediately before treatment, on day 28 of treatment, and 28 days after discontinuing treatment. All 3 biopsy specimens were obtained from each subject immediately before treatment, on day 28 of treatment, and 28 days after discontinuing treatment. Each 6-mm biopsy specimen was bisected: half was placed immediately in neutral buffered 10% formalin and later was paraffin-embedded for histologic sectioning, and the other half was frozen immediately on dry ice and was transferred to a −80°C freezer for later extraction of nucleic acids.

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Informed consent was obtained from subjects participating in this research. The human experimentation guidelines of each participating institution were followed in the conduct of the research.

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Reprints or correspondence: Dr. Dennis M. Walling, Div. of Infectious Diseases, Dept. of Internal Medicine, University of Texas Medical Branch at Galveston, 301 University Blvd., Galveston, TX 77555-0435 (dwalling@utmb.edu).

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1Division of Infectious Diseases, Department of Internal Medicine, and 2Department of Pathology, University of Texas Medical Branch at Galveston, 3Division of Oral and Maxillofacial Pathology, Department of Stomatology, and 4Department of Basic Sciences, University of Texas–Houston, Dental Branch, and 5Bering Omega Community Services, Dental Clinic, Houston
**Histopathologic interpretation.** Tongue biopsy tissue sections (5 μm) were placed on silane-coated glass slides and were stained with hematoxylin-eosin for routine microscopic evaluation. An oral pathologist made a diagnosis after reviewing a minimum of 3 tissue sections. In addition, periodic acid–Schiff staining was done on all tissue samples for the detection of fungal organisms.

**EBV termnii analysis.** DNA (5 μg) from each tongue biopsy specimen was Bam HI digested, was Southern blotted, and was hybridized to a 32P-labeled RNA probe synthesized from the cloned Xho I 1.9-kb EBV genome fragment [12, 13]. Linear EBV termnii, indicative of productive EBV replication, were identified by the presence of a ladder array of bands differing in size by a constant interval of ~500–700 bp, as demonstrated elsewhere for hairy leukoplakia [13, 14].

**EBV transcription analysis.** RNA from tongue biopsy tissue was treated with DNase and was reverse-transcribed into cDNA by use of an oligo-dT primer and avian myeloblastosis virus reverse-transcriptase (Promega). cDNA was amplified by nested polymerase chain reaction, using primers specific for the EBV immediate-early replicative gene (BZLF1), the EBV early replicative gene (BHRF1), and the EBV late replicative gene (gp220). As a control for detection of EBV transcripts potentially originating in B lymphocytes present in the tongue tissue, cDNA was amplified by nested polymerase chain reaction, using primers specific for leukocyte common antigen (CD45) message and B lymphocyte–specific CD19 message. All primers were designed from gene exon sequences flanking introns that are spliced out of the mRNA transcript during translational expression of the gene (table 1) [15–19].

Forty cycles of amplification were done for the initial reaction and for the nested reaction (80 cycles total), using DNA polymerase and buffer (Vent; New England Biolabs) and a thermal cycler (RoboCycler; Stratagene). Amplified products were identified by DNA size, bp

**EBV immunohistochemistry.** Tongue biopsy tissue sections (5 μm) were placed on silane-coated glass slides, were deparaffinized, and were washed with PBS. Slides were pretreated with 3% hydrogen peroxide and citrate antigen retrieval buffer (DAKO). Tissue sections were stained with a murine monoclonal antibody (IgG) to the EBV BZLF1 protein (Clone BZ.1; DAKO). A biotinylated anti–mouse IgG, streptavidin–horseradish peroxidase, and diamino-benzidine were used for staining. Slides were counterstained with Harris hematoxylin for examination by light microscopy.

**EBV in situ hybridization.** Tongue biopsy tissue sections (5 μm) were placed on silane-coated glass slides and were hybridized with a biotin-labeled EBV DNA probe (Bam H1 “V” fragment; Enzo Diagnostics) diluted to 2 ng/μL. Bound probe was detected by use of an in situ hybridization detection system for biotinylated probes (DAKO). Slides were counterstained with nuclear fast red for examination by light microscopy.

**Results**

Productive EBV replication was detected in normal tongue epithelial cells from 2 subjects. Subject 1, who had hairy leukoplakia at enrollment, had a CD4 cell count of 22 cells/mL and an unknown HIV load and was not receiving antiretroviral treatment. Microscopic findings of the first tongue specimen demonstrated the classic features of hairy leukoplakia, including epithelial hyperplasia with shaggy hyperparakeratosis, acanthosis, and koilocyte-like cells in the stratum spinosum (figure 1). Numerous hyphal forms that were morphologically consistent with Candida species also were detected in the superficial cell layer, along with a mild chronic inflammatory cell infiltrate, indicating a Candida superinfection of this hairy leukoplakia lesion.

Molecular evidence of productive EBV replication in this hairy leukoplakia specimen included nuclear expression of EBV replicative protein BZLF1 in koilocyte-like cells of the upper epithelial layer (figure 2), the presence of intracellular EBV DNA in koilocyte-like cells of the upper epithelial layer (figure 3), and the presence of linear EBV genome termnii in the hairy leukoplakia tissue (figure 4). Productive EBV replication was further demonstrated by transcription of EBV replicative genes BZLF1, BHRF1, and gp220 (table 2). During

| Table 1. Reverse-transcriptase polymerase chain reaction primers and products for Epstein-Barr virus (EBV) and human gene transcription analysis of tongue biopsy RNA samples. |
| --- | --- | --- | --- |
| Gene amplified | Initial primers, 5'→3' | Nested primers, 5'→3' | DNA product size, bp | mRNA product size, bp |
| EBV BZLF1 | GCCACATCGTCTCAACAGGG | CCGTCTATTTCAGATGTTTG | 434 | 226 |
| EBV BHRF1 | AACAGCTAGCAGAGAACTGG | ACAAAGCTAGCAGACATTGG | 352 | 176 |
| EBV gp220 | GTGTTGTGTAATACTGTCCTCAAT | TCCAGTTTCTCTGAAATGCTC | 755 | 316 |
| Human CD45 | GCCGATATGGTCGTTGCTGCT | TCCAGTTTCTCTGAAATGCTC | 1009 | 262 |
| Human CD19 | GCTGTTAGCTATTGCTGTTT | No product | No product | 99–581* |

Note: BHRF1, EBV early replicative gene; BZLF1, EBV immediate-early replicative gene.

* Predicted product size depends on posttranscriptional CD45 message processing and variable inclusion of exons 4, 5, and 6.
valacyclovir treatment, hairy leukoplakia resolved, and molecular evidence for EBV replication was absent (table 2).

Hairy leukoplakia did not recur within the 28-day posttreatment study period. Histologic examination of the tongue after treatment again demonstrated a superficial fungal infection with a mild chronic inflammatory cell infiltrate, but there was no evidence of hairy leukoplakia. Molecular evidence for reactivation of productive EBV replication in this tissue included the presence of linear EBV genome termini at quantitatively lower levels than in the previous sample (figure 4) and transcription of EBV replicative genes BZLF1, BHRF1, and gp220 (table 2). In this specimen, CD45 transcription also was detected at trace levels.

Figure 1. Hairy leukoplakia tissue sample exhibiting mild epithelial hyperplasia with shaggy hyperkeratosis and a band of koilocyte-like cells in the upper spinous cell layer (hematoxylin-eosin stain; original magnification, ×200).

Figure 2. Immunohistochemical staining for the Epstein-Barr virus immediate-early replicative protein BZLF1 in a hairy leukoplakia tissue sample. Strong nuclear reactivity within morphologically abnormal koilocyte-like cells and other keratinocytes of the upper spinous cell layer is seen (hematoxylin stain; original magnification, ×200).
levels (table 2) in an exon splicing pattern consistent with T but not B lymphocyte origin (absence of exon 4) [20, 21]. This CD45 transcription likely represents the infiltrating lymphocytes seen histologically, but it is unlikely that the replicative EBV transcripts arose from these T lymphocytes. Staining for BZLF1 protein and EBV DNA was negative for both lymphocytes and epithelial cells in the tissue sections examined from this specimen.

Subject 2 did not manifest hairy leukoplakia at any time during the study period and had a CD4 cell count of 220 cells/mL and an unknown HIV load. Subject 2 was receiving antiretroviral therapy with stavudine, zalcitabine, and indinavir. The gross appearance of the tongue was normal, and none of the histopathologic features of hairy leukoplakia was present in any of the 3 tongue biopsy specimens. However, occasional hyphal forms that were morphologically consistent with Candida species were detected in the superficial cell layer, along with a mild chronic inflammatory cell infiltrate, in the first of the 3 biopsy specimens.

Before valacyclovir treatment, molecular evidence of productive EBV replication in this tongue tissue included nuclear expression of EBV replicative protein BZLF1 in the foci of Figure 3. In situ hybridization for Epstein-Barr virus DNA in hairy leukoplakia tissue sample. Strong nuclear reactivity within morphologically abnormal koilocyte-like cells and other keratinocytes of the upper spinous cell layer is seen (nuclear fast red stain; original magnification, ×200).

Figure 4. Southern hybridization to Epstein-Barr virus (EBV) terminal fragments in hairy leukoplakia tissue sample obtained before valacyclovir treatment (TBX1). A ladder array of bands indicative of productive EBV replication is demonstrated. The quantity of EBV in the sample was estimated to be >50 genome copies/cell, as determined by comparison with standard dilutions of EBV DNA from Raji Burkitt’s lymphoma cell line. EBV terminal fragments were not detected in the normal tongue tissue obtained during valacyclovir treatment (TBX2). EBV terminal fragments were detected as a ladder array of bands in the normal tongue tissue obtained after valacyclovir treatment (TBX3), after longer exposure of the autoradiograph (right panel).
normal superficial epithelial cells (table 2), intracellular expression of EBV DNA in foci of normal superficial epithelial cells (table 2), and the abundant presence of linear EBV genome termini (figure 5). Productive EBV replication was further demonstrated by transcription of EBV replicative genes BZLF1, BHRF1, and gp220 (table 2). In this specimen, CD19 transcription also was detected at trace levels (table 2). This result raises the possibility of EBV transcription originating from EBV-infected B lymphocytes infiltrating this tissue. However, the immunohistochemical and in situ hybridization stains failed to demonstrate EBV expression in any of the tissue lymphocytes, while clearly identifying EBV replication in numerous epithelial cells. During valacyclovir treatment, molecular evidence for EBV replication was absent except for gp220 transcription (table 2).

Twenty-eight days after treatment, the tongue epithelium remained histologically normal (figure 6). Molecular evidence of productive EBV replication in this normal tongue tissue again included transcription of EBV replicative genes BZLF1, BHRF1, and gp220 (table 2), nuclear expression of EBV replicative protein BZLF1 in the foci of normal superficial epithelial cells (figure 7), intracellular expression of EBV DNA in the foci of normal superficial epithelial cells (figure 8), and the presence of linear EBV genome termini (figure 5) at a level quantitatively similar to that of the hairy leukoplakia sample from subject 1.

### Discussion

Productive EBV infection is a sequential process of viral transcriptional transactivation, genome replication, virion assembly, genome packaging, and release of enveloped virus particles from the cell. In this study, EBV transcriptional transactivation was demonstrated by immediate-early (BZLF1) and early (BHRF1) gene expression [10, 22–24]. EBV genome replication was demonstrated by DNA in situ hybridization [11, 25, 26]. EBV genome packaging was demonstrated by the presence of linear genome termini generated by packaging-dependent

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**Table 2.** Summary of productive Epstein-Barr virus (EBV) replication in tongue biopsy tissues from 2 subjects before, during, and after treatment with valacyclovir (VACV), as demonstrated by transcription of various EBV replicative genes.

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<tr>
<th>Subject, time of biopsy</th>
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<th>Tissue histology</th>
<th>Genome termini</th>
<th>BZLF1 by IHC</th>
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**NOTE.** BHRF1, EBV early replicative gene; BZLF1, EBV immediate-early replicative gene; HLP, hairy leukoplakia; IHC, immunohistochemistry; ISH, in situ hybridization; RT-PCR, reverse-transcriptase polymerase chain reaction; +, present; −, absent.

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**Figure 5.** Southern hybridization to Epstein-Barr virus (EBV) terminal fragments in normal tongue tissue obtained before the subject received valacyclovir treatment (TBX1). A ladder array of bands indicative of productive EBV replication is demonstrated. EBV terminal fragments were not detected in normal tongue tissue obtained during valacyclovir treatment (TBX2) but were present in normal tongue tissue obtained after valacyclovir treatment (TBX3). The EBV quantity in this normal tongue tissue was estimated to be 5–50 genome copies/cell, as determined by comparison with standard dilutions of EBV DNA from Raji Burkitt’s lymphoma cell line.
cleavage of the concatemeric viral genome during insertion into the nucleocapsid [12, 27, 28]. Expression of the EBV envelope glycoprotein gp220 suggests virion processing and maturation through the cellular Golgi apparatus immediately before plasma membrane budding [29, 30]. Proof of productive EBV replication would have been further strengthened by demonstrating extracellular virion structures in the tissue by electron microscopy, but sufficient tissue was not available from this study. Other investigators have reported herpesvirus-like virion structures by electron microscopy in both hairy leukoplakia and normal tongue tissues from immunocompromised individuals [10, 31–33].

Figure 6. Essentially normal tongue tissue exhibiting only mild epithelial hyperplasia and minimal chronic inflammation, likely explained by *Candida* superinfection of the tongue epithelium, as detected by periodic acid–Schiff staining (data not shown). Although spongiotic cells were observed in the spinous cell layer, koilocyte-like cells were not found in this tissue (hematoxylin-eosin stain; original magnification, ×100).

Figure 7. Immunohistochemical staining for the Epstein-Barr virus immediate-early replicative protein BZLF1 in normal tongue tissue. Strong nuclear reactivity within foci of morphologically normal superficial keratinocytes is seen (hematoxylin stain; original magnification, ×200).
Productive EBV replication is believed to be etiologically important to the pathogenesis of hairy leukoplakia [11]. EBV replication in normal epithelium involved individual foci of epithelial cells rather than large continuous regions of epithelium, as seen with hairy leukoplakia. However, the measured quantities of EBV replication were similar between the normal tongue and that with hairy leukoplakia (figures 4 and 5), and the intensity of staining for BZLF1 expression and EBV genome replication appears to be quantitatively similar in the morphologically normal epithelial cells and in the koilocyte-like cells of hairy leukoplakia (figures 2, 3, 7, and 8). Antiviral therapy completely abrogated EBV replication in tongue epithelium, eliminating the hairy leukoplakia when it was present [11]. Thus, productive EBV replication is necessary but insufficient for the pathogenesis of hairy leukoplakia. Additional cofactors likely are needed to produce the koilocyte-like epithelial cell morphology and other histopathologic features of the lesion. Identification of these cofactors, such as specific latency-associated EBV gene products expressed in hairy leukoplakia [34, 35], could have implications for understanding EBV pathogenesis in other EBV-associated epithelial diseases, such as nasopharyngeal carcinoma.

In both hairy leukoplakia and normal tongue epithelium, EBV replication occurs in the upper-layer epithelial cells that are undergoing terminal differentiation, apoptosis, and desquamation [26, 36, 37]. This observation adds further support to the hypothesis that EBV replication is dependent on the differentiation state of the epithelial cell [38]. Productive EBV replication in epithelial cells may not be “lytic” in the same sense that this term is applied to EBV replication and induction of cell death in undifferentiated Burkitt’s lymphoma cell lines and B lymphoblastoid cell lines in vitro. Consequently, intentional induction of EBV replication may not be therapeutically useful for undifferentiated EBV latently infected epithelial malignancies, as has been proposed for EBV-associated lymphoid malignancies [39].

This demonstration of EBV replication in normal tongue epithelial cells suggests that the tongue may be a source of the EBV secreted into the saliva of HIV-infected individuals without hairy leukoplakia [40, 41]. In addition, productive replication in normal tongue epithelium should be investigated as a potential source of the salivary EBV detected in mononucleosis patients [2, 3] and in otherwise healthy individuals persistently infected with EBV [42, 43]. Although this study was limited to examination of the tongue, other oral sites may also support productive EBV replication in normal epithelial cells [25, 44–46]. Productive replication in desquamating oral epithelial cells would seem to be an efficient strategy for EBV transmission.

Finally, this demonstration of persistent EBV infection in normal oral epithelial cells sheds new light on the continuing debate about the potential role of epithelial tissues in EBV persistence [4, 5, 47]. It is possible that both lymphoid and epithelial tissues can support long-term, persistent EBV infection. However, these data must be interpreted in the context of the host-parasite relationship. HIV infection either unmasks intrinsic properties of EBV that are important for its persistence and pathogenesis in all hosts or expands the biologic repertoire and host cell range of EBV beyond that normally required for persistence.
and transmission. Thus, extending these observations to non-immunocompromised individuals is essential to understanding the ultimate importance of the oral epithelium in EBV persistence and transmission.

References


