Expression of proteins encoded by Epstein–Barr virus trans-activator genes depends on the differentiation of epithelial cells in oral hairy leukoplakia

(EPSTEIN–BARR VIRAL ANTIGENS/IMMUNOSTAINING/HUMAN IMMUNODEFICIENCY VIRUS INFECTION)

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ABSTRACT The Epstein–Barr virus (EBV) immediate early gene product BZLF1 was localized by indirect immunofluorescence to the cytoplasm of the basal epithelial layer at the lateral border and dorsum of tongue in human immunodeficiency virus-infected and -seronegative patients. Two biopsies of oral hairy leukoplakia revealed a sporadic cytoplasmic staining of the BHRF1 and BRLF1 gene products in the basal epithelial layer. The widespread presence of BZLF1 in the basal epithelial layer indicated that this cell layer contained EBV DNA and was probably directly infected by EBV. Nuclear localization of the immediate early and early gene products BZLF1, BHRF1, BRLF1, and BMLF1 was limited to oral hairy leukoplakia in human immunodeficiency virus-seropositive patients and revealed a codistribution with the virus capsid antigen. Our results indicate that the epithelium of the tongue is a potential reservoir for EBV and that in heavily immunocompromised patients EBV may move from the cytoplasm to the nucleus with increasing differentiation and be concentrated there during the terminal differentiation of epithelial cells at the lateral border and dorsum of tongue.

The Epstein–Barr virus (EBV) is a ubiquitous herpesvirus with a linear double-stranded DNA genome, which has been completely sequenced (1, 2). It causes polyclonal immortalization of human B cells both in vivo and in vitro (for review, see ref. 3) and persists within latently infected cells as multiple, covalently closed circular episomes (4). EBV infection of lymphoid cells is primarily latent as indicated by the absence of antibodies to early EBV-encoded antigens, such as p54 and p138 in healthy EBV-seropositive individuals (5). In latently infected cells viral gene expression is restricted to a few viral products: the EBV nuclear antigens (for review, see ref. 6), the BNLF1 membrane protein (latent membrane protein; ref. 7), EBV-encoded small RNAs (8), and terminal protein (9). The cellular and viral factors that determine whether EBV infection is latent or productive are mainly unknown and depend on the host cell (10).

The concept of strict B-lymphocyte tropism has been central to discussion of the biology of EBV since the discovery of this herpesvirus. The observation that epithelial cells in nasopharyngeal carcinoma are latently infected with EBV and that lytic expression of EBV occurs in epithelial cells of the parotid gland (11) and other sites of the human body (12) clearly points to a complex biology of this herpesvirus.

Viruses binding to B cells occurs via a specific interaction between the major envelope glycoprotein gp340 and the C3d receptor molecule (CD21; refs. 13 and 14) and it seems that the specificity of gp340–CD21 binding plays a major role in determining the viral B lymphotropism (15).

The presence of EBV in the upper two-thirds of the epithelium in oral hairy leukoplakia (OHL) was originally described by Greenspan et al. (16) and later confirmed by ultrastructural studies (17), immunohistochemistry, and in situ hybridization (18–20).

The means by which EBV gains access to epithelial cells in general and the questions of which epithelial cell layer in OHL could be infected by EBV and of how the virus reaches the replicating cells in OHL have been the subject of numerous studies (15, 21–24). Different modes of infection have been suggested: Receptor molecules similar but not identical to CD21 have been reported from undifferentiated epithelial cells of the oropharynx (22, 25). It is possible that basal epithelial cells in OHL could be infected through such receptors or via cell fusion (21) by EBV-infected B lymphocytes from peripheral blood. Production of EBV in OHL could be initiated later as a consequence of cellular differentiation (26). It has been shown that human epithelial cells of normal nasopharynx and tonsil (15), as well as of the upper and middle spinous cell layer of OHL (23), express a 200-kDa surface molecule that is antigenetically related to, but not identical with, the CD21 antigen of B cells (15). It is possible that this cell layer, where lytic expression of EBV is observed in OHL, could be infected by free virus in the saliva from the parotid gland. But this receptor molecule, which is detected by monoclonal antibody HB5, is not limited to the lateral border of tongue and is also present in other parakeratinized areas—i.e., gingiva, soft palate (23)—where lytic expression of EBV has so far not been observed.

To get further insight into the mode and time of infection of epithelial cells we used antibodies to regulatory and structural gene products of EBV on biopsy material of HIV-seronegative and HIV-infected patients.

METHODS

Biopsies. Thirty-three biopsies (Table 1) were taken under local anesthesia for diagnostic reasons or during surgery for other reasons. Biopsies of uninvolved mucosa were taken in a systematic manner from volunteers after information about the proposed study. Immediately after removal biopsies were divided. One portion was fixed with 10% formalin and embedded in paraffin for routine histology (hematoxylin/eosin and periodic acid/Schiff staining). The other portion was frozen in liquid nitrogen and stored at −75°C.

Abbreviations: EBV, Epstein–Barr virus; OHL, oral hairy leukoplakia; HIV, human immunodeficiency virus; VCA, viral capsid antigen; ORF, open reading frame.

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All patients were examined for human immunodeficiency virus (HIV) infection by ELISA and Western blotting. The clinical status was diagnosed according to the Centers for Disease Control definition (27). All patients were examined for EBV infection and demonstrated IgG and IgM anti-viral capsid antigen (VCA) antibodies except the youngest, a 5-year-old patient, who did not show anti-EBV IgG and IgM antibodies.

For indirect immunofluorescence, cryostat sections (5 µm) were air dried at room temperature for 2 h and fixed with chloroform/acetone for 5 min at 5°C. Sections were incubated first with primary antibody (dilution, 1:20) and then with tetramethyl-rhodamine isothiocyanate (TRITC)-conjugated goat anti-rabbit or fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse second antibody (dilution, 1:20; Jackson ImmunoResearch). After each step sections were washed three times with phosphate-buffered saline, pH 7.2. The localization of BZLF1 and BcLF1 (p150) included double-staining experiments. These sections were incubated first with antiserum to BZLF1 followed by the TRITC-conjugated second antibody and then with monoclonal antibody to BcLF1 followed by the FITC-conjugated goat anti-mouse IgG (dilution, 1:20; Jackson ImmunoResearch).

Controls using second antibodies only were included for every biopsy. Immunostaining was visualized with a Leitz Orthoplan microscope and photographed on Ilford HP5 films.

**Antibodies.** A monoclonal antibody specific for open reading frame (ORF) BcLF1 was produced against authentic viral protein by inoculation of EBV-VCA positive P3HR-1 cells (W.J., Helga Mairhofer, and H.W., unpublished results) and was demonstrated to be highly specific on semithin cryostat sections of OHL (17). Sera against the proteins expressed by ORFs BZLF1 (amino acids 90–166 of the ORF; exon 1), BMLF1 (amino acids 1–165), BRLF1 (amino acids 400–600), and BHRF1 (amino acids 24–138) have been derived using β-galactosidase fusion proteins as described (28, 29). A second polyclonal antiserum specific for amino acids 175–189 of BZLF1 (exon 2) was raised by inoculation of synthetic peptides in rabbits. A c-fos antiserum was kindly provided by R. Müller (Marburg, F.R.G.).

**RESULTS**

**Biopsies from HIV-Seronegative Patients.** All biopsies were completely negative for BRLF1, BMLF1, and BcLF1 (p150) and for the antiserum against c-fos. Four biopsies from normal tongue (three males and one female) and two from smokers’ leukoplakia revealed an intense cytoplasmatic fluorescence for BZLF1 in the basal epithelial layer (Fig. 1). The presence of BZLF1 in three of these biopsies from normal tongue was not limited to the lateral border of tongue (Fig. 2). The fluorescence for BZLF1 was not present in all basal epithelial cells, extensive areas were negative (Fig. 3). Two biopsies from smokers’ leukoplakia and three biopsies from normal tongue, including multiple sections from an EBV-seronegative five-year-old patient, did not show any fluorescence for BZLF1 in the basal epithelial layer at the lateral border of tongue. The antibodies against exons 1 and 2 of BZLF1 revealed similar results.

**Biopsies from HIV-Infected Patients.** Immunolabeling for antibodies against the ORFs BcLF1 (VCA), BRLF1, BMLF1, and BHRF1 was limited to OHL biopsies (Fig. 3). The antiserum against c-fos did not show any immunoreaction as was also noted in HIV-seronegative patients.

**BcLF1** (p150). The VCA was observed only in the nuclei of epithelial cells and in the extracellular space in the upper stratum spinosum (Fig. 4A).

**Fig. 1.** Normal oral mucosa of the dorsum of the tongue of a HIV-seronegative patient. Strong cytoplasmatic fluorescence for BZLF1 is shown in the basal epithelial layer. (x600.)

**Fig. 2.** Schematic drawing of the dorsum and lateral border of tongue. Areas that were found to be positive for BZLF1 in the cytoplasm of the basal epithelial layer in our biopsy material are indicated.
**DISCUSSION**

*In vitro* models have shown that latent EBV infection is controlled by complex interactions between B and T cells as well as their secreted products, immunoglobulins, interferons, and interleukins. An important component of the inhibition of lytic expression in vivo seems to be cooperation of HLA-restricted cytotoxic T cells and the CD4+ subset (30, 31). This EBV-directed immune elimination does not reach all sites of the human body and it has been shown that cells of the ductal epithelium of the parotid gland can undergo a complete lytic cycle in nonimmunocompromised patients (11).

The replication of EBV in epithelial cells of the stratum spinosum at the lateral border of tongue in immunocompromised patients is an obvious sign that the immune system has lost the ability to eliminate cells expressing other than latent gene products of EBV. Recent individual reports have shown that the lytic cycle of EBV replication in OHL is not limited to immunocompromised patients under HIV infection (32). OHL is therefore not a specific sign of HIV infection, but is related to immunosuppression in general (33).

We have shown the presence in HIV-seropositive patients of the EBV immediate early gene product BZLF1 and occasionally of BHRF1 and BRLF1 in the cytoplasm of the basal epithelial layer of the tongue. These findings indicate that this cell layer contains EBV genomes and is probably directly infected by EBV. In particular the demonstration of BZLF1 in immunocompromised HIV-infected as well as HIV-seronegative patients indicates further that the tongue is a potential reservoir for EBV.

The patchlike distribution of BZLF1-specific cytoplasmic fluorescence in the basal epithelial layer in HIV-seronegative patients suggests that this epithelial layer is probably not continuously infected. The presence of EBV in basal epithelial cells could result from a horizontal transmission of viral genomes during the proliferation of epithelial progenitor cells.

The increase of BZLF1-positive cells in the basal epithelial layer of OHL may be a consequence of the general loss of the immune system to eliminate cells expressing other than latent

**FIG. 3.** In HIV-seronegative patients, BZLF1 was localized to the cytoplasm of the basal epithelial layer at the lateral border or dorsum of tongue, with the immunolabelling showing as a patchlike distribution and extensive areas between labeled cells remaining unstained. In OHL patients, BZLF1 fluorescence was observed in the cytoplasm of nearly the entire basal epithelial layer. Nuclear staining for immediate early and early antigens as well as virus structural proteins (BcLF1, VCA) indicates the limitation of the lytic cycle of EBV to the upper stratum spinosum and the overlying ballooned keratinocytes. All proteins examined revealed a nuclear codistribution in this location.

**BZLF1.** All OHL biopsies showed an intense fluorescence for BZLF1 throughout almost the entire basal epithelial layer (Fig. 4B). The adjacent stratum spinosum was completely negative. In the upper stratum spinosum and in the ballooned keratinocytes a nuclear fluorescence was noted, and double immunofluorescence experiments revealed in this area a nuclear codistribution of BZLF1 and BcLF1 (Fig. 4).

Only one biopsy from normal lateral tongue (Centers for Disease Control stage CIII) showed intense cytoplasmatic fluorescence in the basal epithelial layer. The other two biopsies from lateral tongue were negative as was the orthokeratinized gingiva. The antibodies against the exons 1 and 2 gave similar results.

**BRLF1.** OHL biopsies showed a nuclear fluorescence for BRLF1 in the upper stratum spinosum and in the overlying ballooned keratinocytes (Fig. 3). Two patients revealed a sporadic but strong cytoplasmatic fluorescence in epithelial cells of the basal epithelial layer.

**BMLF1.** BMLF1 fluorescence was observed only in the upper stratum spinosum of OHL biopsies and showed a nuclear codistribution with BcLF1 fluorescence (Figs. 3 and 5).

**BHRF1.** BHRF1 showed as a nuclear fluorescence in the upper stratum spinosum and in the overlying ballooned keratinocytes of OHL biopsies as was observed for BcLF1 (Fig. 3). In two patients a further cytoplasmic staining was noted in the basal epithelial layer.
gene products as indicated by the lytic cycle of EBV expression in the stratum spinosum. When this is interpreted as a defect in the elimination of cells entering a lytic cycle of viral replication one can also assume that further epithelial cells could be infected through EBV-expressing B lymphocytes from peripheral blood.

We have shown that the presence of the EBV immediate early gene product BZLF1 in the cytoplasm of basal epithelial cells is not limited to the lateral border of the tongue. Recent clinical findings underline these results with the description of lytic expression of EBV at the dorsum of tongue (34, 35). To exclude cross-reactivity between BZLF1 and c-fos in basal epithelial cells (36) we used antibodies against different exons of BZLF1 and an antibody to c-fos. Our results exclude a cross-reactivity with c-fos in our biopsy material. A further hint for the specificity of the immunohistochemical detection of BZLF1 in the basal layer was the detection of BHRF1 and BRLF1 in this location. The fact that BHRF1 was observed in two samples is in agreement with observations by Austin et al. (37) of transient expression of this protein in lymphocytes. However, our observation does not necessarily support direct involvement of this gene in induction of the lytic cycle. The sporadic observation of BRLF1 may reflect a tissue-specific regulatory event and low levels of expression.

In the stratum spinosum, where BZLF1 was detected in the nuclei, late viral genes are expressed in regions positive for BZLF1. It has been shown that expression of BZLF1 is sufficient to disrupt latency (38, 39). Our findings strongly suggest that a nuclear localization for BZLF1 (29) is necessary for its trans-activating function on other EBV genes (39–42). BZLF1 may move from cytoplasm to nucleus with increasing differentiation in immunocompromised patients and the mechanism may be similar to that of nuclear factor κB in HIV-infected T cells (43), which is present in an inactive form due to formation of a complex with a cellular protein in nonactivated cells and changes its location in response to external stimuli. Alternatively, a cellular protein may be required for activation of BZLF1, such as the cellular protein E2F, which activates the adenosivirus-encoded E1A protein in adenovirus-infected cells (44).

Various studies employing in situ hybridization have failed to detect low copy numbers of EBV-specific nucleic acids in the basal epithelial layer of OHL because of the limited sensitivity of this technique (18, 45). Independent proof of EBV infection of the basal layer, in addition to the demon-
stration of BZLF1, is desirable. But there are technical constraints at this time to block otherwise widely used technical approaches. The polymerase chain reaction, which is a highly sensitive technique to detect EBV in HIV-infected patients (45), can in our opinion not be applied for this question, because of the difficulties to extract only the basal layer of the stratified epithelium and to exclude contamination with free virus shedding from oropharyngeal mucosa.

In conclusion, our findings show that the basal epithelial cell layer of the lateral border and the dorsum of tongue is probably a potential reservoir for EBV in HIV-seropositive and in nonimmunocompromised HIV-seronegative individuals. In heavily immunosuppressed patients latently EBV-infected epithelial cells may be coactivated during terminal differentiation of the epithelium. Due to the regular stratification of the epithelium OHL is an excellent model to study the host cell-dependent activation of EBV genes using antibodies to different gene products until more-sensitive techniques for in situ hybridization are available.

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