Epstein-Barr Virus Infection in Ex Vivo Tonsil Epithelial Cell Cultures of Asymptomatic Carriers

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Epstein-Barr virus (EBV) is found frequently in certain epithelial pathologies, such as nasopharyngeal carcinoma and oral hairy leukoplakia, indicating that the virus can infect epithelial cells in vivo. Recent studies of cell lines imply that epithelial cells may also play a role in persistent EBV infection in vivo. In this report, we show the establishment and characterization of an ex vivo culture model of tonsil epithelial cells, a likely site for EBV infection in vivo. Primary epithelial-cell cultures, generated from tonsil explants, contained a heterogeneous mixture of cells with an ongoing process of differentiation. Keratin expression profiles were consistent with the presence of cells from both surface and crypt epithelia. A small subset of cells could be latently infected by coculture with EBV-releasing cell lines, but not with cell-free virus. We also detected viral-DNA, -mRNA, and -protein expression in cultures from EBV-positive tonsil donors prior to in vitro infection. We conclude that these cells were either already infected at the time of explantation or soon after through cell-to-cell contact with B cells replicating EBV in the explant. Taken together, these findings suggest that the tonsil epithelium of asymptomatic virus carriers is able to sustain EBV infection in vivo. This provides an explanation for the presence of EBV in naso- and oropharyngeal pathologies and is consistent with epithelial cells playing a role in the egress of EBV during persistent infection.

Epstein-Barr virus (EBV) is a ubiquitous, persistent human herpesvirus whose tropism for B lymphocytes is well established. However, some reports have also suggested an epithelial-cell tropism for the virus based on claims of having identified EBV-infected pharyngeal epithelial cells in vivo (46). This led many investigators to propose that the mucosal epithelium of the nasopharyngeal region was the site of EBV persistence and that the infection of B cells was not critical for viral persistence (1, 41, 42, 61). Subsequently, however, these findings were challenged based on the failure of other investigators to reproduce them (25, 35) and the mounting body of evidence implicating memory B cells as the site of EBV persistence (4, 13, 17, 51, 52). Specifically, it has not been possible to reproducibly detect infected epithelial cells in cross sections of tonsils or exfoliated epithelial cells in the saliva of acutely infected patients (25, 35). Even though these studies did not rule out low levels of infected cells, they encouraged the belief that EBV does not normally infect healthy epithelial cells in vivo. Nevertheless, EBV is 100% associated with naso- and oropharyngeal epithelial pathologies, such as nasopharyngeal carcinoma (NPC) (3) and oral hairy leukoplakia (OHL) (18). In NPC, the epithelial cells are predominantly latently infected, whereas in OHL, an AIDS-associated lesion of the oral epithelium, infectious virus is produced by epithelial cells in large amounts (58). From these findings, it is clear that EBV can latently infect and replicate in oral- and nasopharyngeal epithelia in vivo. This raises the possibility that these lesions may be amplified versions of infected healthy epithelium.

A recent in vitro breakthrough in understanding the infection of oropharyngeal epithelial cells was provided when Bl integrin was identified as a crucial component of the receptor that mediates EBV infection in subsets of oral epithelial cell lines (54). Other in vitro findings provided a mechanistic explanation for this proposed dual tissue tropism (8, 10). Virions produced by B cells in vitro have glycoprotein expression patterns that favor infection of epithelial cells and vice versa. This could result in a preferential exchange of EBV between the tissues. If EBV in vivo is transmitted back and forth between epithelial cells and B cells, the tissues must be in close proximity to one another. Tonsil reticulated crypt epithelium (37, 38) is one such site where EBV-infected B cells are in intimate contact with neighboring epithelium (2, 34). Great improvements have been made in understanding the role of B cells in EBV persistence in vivo due to the accessibility of infected primary B lymphocytes in the blood and lymph nodes (51, 52) and the availability of an in vitro infection system (26). This work has established the importance of B cells in the tonsil lymphoepithelium as a critical site for the establishment of latent persistent infection and for replication of the virus (51). In contrast, evidence that nasopharyngeal epithelial cells play a role in EBV persistence and/or replication in healthy carriers is still lacking.

One possible role for the epithelium in the nasopharynx is as the first tissue that becomes infected after exposure to the virus, because EBV is transmitted via saliva (19). Lytic replication in epithelial cells could then act as a transit route or an amplification step prior to infection of the main target, B cells residing in the underlying lymphoepithelium. Alternatively, epithelial cells could be used to transit and release virus, produced from infected B cells in the lymphoepithelium, back into saliva. This kind of dual tissue tropism strategy is consistent with the behavior of other ubiquitous herpesviruses (43), and...
in vivo evidence for such an infection route has recently been provided for simian immunodeficiency virus in monkeys (50).

The failure to detect infected epithelial cells in vivo and the lack of an accurate primary in vitro nasopharyngeal epithelial culture model has greatly hampered research in understanding the role these cells might play in persistence. For these reasons, we set out to establish and characterize a primary tonsil epithelial culture system that retains the heterogeneity of the tissue found in vivo. We have developed early ex vivo tonsil epithelial-cell cultures that express keratin markers indicative of crypt and surface tonsil epithelia. A subset of these cells is reproducibly infectible in vitro by coculture with EBV producer cells. Surprisingly, evidence of EBV infection was detected prior to the addition of exogenous virus in cultures from EBV-positive tonsils even when maintained in the presence of acyclovir. These observations suggest that the epithelial cells were infected either in vivo or rapidly after explantation by virus already present in the tissue explants. To our knowledge, these findings are the first to describe EBV infection of healthy tonsil epithelial cells derived from asymptomatic virus carriers. Our data support the model of dual epithelial-lymphoid tropism for the virus in vivo and the possibility that healthy tonsil epithelium in vivo may play a role in transmission of the virus as part of the viral life cycle and suggest that EBV can play an initiating role in associated epithelial lesions, such as NPC and OHL.

MATERIALS AND METHODS

Primary cultures and cell lines. Tonsillectomies were performed at Massachusetts General Hospital on 6- to 14-year-old patients because of obstructed-breathing disorders. Tonsils were kept on ice in PBSAPSF (1 X phosphate-buffered saline [PBS]-0.5% bovine serum albumin-PennStrepFungizone; Gibco) and were processed between 2 and 6 h after surgery. The epithelial culture method we applied is a modified version of the method for culturing epidermal keratinocytes developed by Lindberg and Rheinwald (30, 40, 44). Epithelial sections of the tonsil exterior were separated from soft tissue (lymphocytes) by scraping the blunt side of a razor blade over the tissue sections. The tissue sections were washed in cold PBSAPSF to remove nonepithelial stroma (mostly lymphocytes) and debris. The tissue found in vivo. We have developed early ex vivo tonsil epithelial-cell cultures that express keratin markers indicative of crypt and surface tonsil epithelia. A subset of these cells is reproducibly infectible in vitro by coculture with EBV producer cells. Surprisingly, evidence of EBV infection was detected prior to the addition of exogenous virus in cultures from EBV-positive tonsils even when maintained in the presence of acyclovir. These observations suggest that the epithelial cells were infected either in vivo or rapidly after explantation by virus already present in the tissue explants. To our knowledge, these findings are the first to describe EBV infection of healthy tonsil epithelial cells derived from asymptomatic virus carriers. Our data support the model of dual epithelial-lymphoid tropism for the virus in vivo and the possibility that healthy tonsil epithelium in vivo may play a role in transmission of the virus as part of the viral life cycle and suggest that EBV can play an initiating role in associated epithelial lesions, such as NPC and OHL.

PCR. Reverse transcription (RT)-PCR for latent genes was performed as previously described (5, 20). Briefly, RNA was purified from 2 X 10^6 epithelial cells and 5 X 10^5 lymphocytes using Trizol reagent (Invitrogen) as described by the manufacturer. Lymphocytes from EBV-negative tonsils, the EBV-negative BJAB B-cell line, and the EBV-positive IB4 B-cell line were used as controls. cDNA was prepared as described previously (5, 20), except that the 20-μl cDNA mixture was not ethanol precipitated but brought up to 100 μl with high-perfomance liquid chromatography H2O and used directly. PCR was performed on the synthesized cDNA for EBNA1-A-K, EBNA1-Q (EBNA1 from the Q promotor), EBNA2, LMP1 (latent form), and LMP2a. The reaction was carried out in a final volume of 50 μl in a mixture consisting of 50 mM KCl, 20 mM Tris (pH 8.3), 5 mM MgCl2, 0.2 mM deoxynucleoside triphosphates, and 0.2 mM (each) amplifiers (final concentrations). The exception was LMP1, for which 3.0 mM MgCl2 was used. The amplifiers and PCR strategies are described in detail elsewhere (5, 20). The PCR products were visualized by Southern blotting as described previously (33). Probes for blotting were made from PCR products derived from the IB4 cells.

Coculture and in vitro infection. EBV-A and EBV-B cells and IB4- and B95-8 cells were treated with 20 ng of tetradecanoyl phorbol acetate (Sigma)/ml and 3 ng butyric acid (Sigma) for 1 to 4 days, while Akata cells were cross-linked with 100 μg of Fab immunoglobulin G (IgG) (Jackson Laboratories)/ml overnight to induce virus release. Cell suspensions were centrifuged (5 min at 3,000 x g), and the supernatants were filtered through a 0.45-μm-pore-size filter. One milliliter of supernatant (cell-free virus) was added to 2 X 10^5 epithelial cells in six-well plates in 3 ml of medium. Daudi cells were incubated for 1 h in undiluted and 10-fold-diluted supernatants, washed, and placed in culture. For infection through cell-to-cell culture, producer cells were cultured 20% acyclovir, washed, and resuspended in SFM. Virus-releasing cells were added to six-well plates and incubated with 1 X 10^3 to 2 X 10^3 day 7 to 10 subconfluent layers of primary epithelial cells at a 1:1 or 2:1 ratio. Floating cells were removed at various times points (days 1 to 3) and postinfection by washes, and epithelial cells were transferred to culture slides 3 days postinfection and prepared for staining.

Staining. Cells were cultured as described and replated at various time points (typically days 3 to 10) in two- and eight-well chambered slides (Costar) in SFM. Cytosins of IB4 (an EBV-positive cell line) and BJAB (an EBV-negative cell line) cells were used as controls for viral-protein detection. B-cell (CD21) and epithelial marker (Ber-ep4) expression. Cytosins and culture slides were treated the same way in all staining procedures. For immunohistochemistry (HC), cells were fixed and permeabilized for 10 to 15 min with 100% chilled acetone. For immunofluorescence (IF), cells were fixed and permeabilized for 30 min at room temperature in 4% paraformaldehyde, followed by 10 min with 0.5% Triton X-100 in phosphate-buffered saline (PBS) at 4°C. The slides were subsequently blocked with appropriate serum for 1 h at room temperature prior to indirect staining. For indirect IF staining of viral proteins, the cells were incubated with primary monoclonal antibodies: LMP1-S12 (dilution, 1:5,000), EBNA1, viral capsid antigen (VCA), and EAD (dilution, 1:1000) (gifts of J. Middeldorp) and BZLF1 (dilution, 1:10) (Dako) at room temperature for 30 min and then washed three times in PBS. Secondary goat anti-mouse Alexa 488 and Alexa 594 antibodies (dilution 1:5,000) (Molecular Probes, Breda, The Netherlands) were incubated for 1 h in the dark. The slides were washed with PBS (three times) and examined with a fluorescence microscope (Nikon E400). For HC and IF of cellular proteins, primary antibodies to keratin 8 (K8), K18, K8/K18, K10, K19, K13, involucrin, filaggrin, CD21, and AE1/AE3 (pankeratins) (Neomarkers, Fremont, Calif.) were treated with 1:200 dilution and incubated for 45 min at room temperature. For HC, the slides were developed with an LSAB detection kit (Dako) used according to the manufacturer's instructions, and the slides were visualized using a light microscope (Nikon TE2000). For IF, the slides were developed as described above.
RESULTS

Culture and characterization of primary tonsil epithelial cells. The culture process was performed in three phases. The first culture phase, DMEM-F12 plus 10% FBS with a high (1 mM) calcium concentration, is designed to stimulate the migration and adherence of epithelial cells out of the small tonsil tissues to the culture dish. The second phase, SFM with low (0.1 mM) calcium plus defined KGF, ensures the elimination of lymphocytes and fibroblasts through cell death but promotes epithelial-cell viability and growth. In this phase, keratinocyte growth and migration are still supported by the existing tissue explants (40). In the third phase (SFM plus KGF), the explants are gently removed and the epithelial cells are replated to another dish at ~60% confluency. Cells with clear epithelial morphology started to migrate on days 3 to 5 in culture, and on days 5 to 8, colonies of various sizes surrounded most individual tissue explants. The details are described in Materials and Methods. Figure 1 shows a schematic and representative photographs of the three culture phases. In general, the primary epithelial cells remained viable for up to 4 weeks in culture.

To characterize the purity, heterogeneity, and differentiation stage of the cultured ex vivo tonsil epithelial cells, we performed HC and IF staining for epithelial and lymphoid markers. Day 7 to 8 cultures were isolated and plated at ~80% confluency on culture slides and used for staining the next day. One hundred percent of the cells stained positive for AE1/AE3 (pankeratinocyte marker) but were completely negative for CD21 (pan-B-cell marker) (Fig. 2A, B, and C), indicating that the cultures were of ~100% epithelial lineage. Similar results were obtained when the cells were analyzed by flow cytometry. The absence of keratin 10 and filagrin expression as early as day 7 and later (not shown) indicated that the cells had not undergone growth arrest and were of the nonkeratinizing squamous epithelial lineage (32). Individual epithelial cell types can be distinguished based upon expression of differentiation markers and morphology (30, 32). A subset of morphological large epithelial cells appeared in the cultures over time (Fig. 1, day 7 versus day 10). These cells were epithelial surface antigen (ESA) negative (early differentiation marker [6]), involucrin positive (late differentiation marker [56]), and keratin 13 negative (early differentiation marker) and had a large cytoplasm/nucleus ratio (Fig. 2E, I, and J). This expression profile is consistent with differentiated squamous epithelial cells (32, 56), suggesting that a process of terminal differentiation was ongoing in the cultures. Occasionally epithelial cells tended to round up spontaneously and detach from the dish, a common behavior of terminally differentiating primary epithelial cells in vitro (9). Positive staining of these cells for involucrin confirmed that they were differentiated (data not shown).

Most epithelial tissues are complex, and individual keratin protein expression can be used to define the origin of epithelial cells (30). Tonsil epithelium is comprised of stratified surface and reticulated crypt epithelia (11, 38). In situ staining of tonsil epithelium suggests that expression of the simple epithelial keratins K18 and K8 is limited to the crypt epithelial cells (11). We detected variable K8 and K18 expression early in our ex vivo tonsil cultures, suggesting that a subpopulation of epithelial cells in our cultures were of crypt epithelial lineage (Fig. 2D and H). In addition, we detected variable K19 expression, another potential tonsil crypt lineage marker (11). The variable expression of K19 in the cultures was particularly apparent by immunofluorescence staining compared with pancytokeratin expression (Fig. 2A, B, F, and G) (11, 32). In summary, our culture method produces heterogeneous squamous epithelial cultures in which differentiation appears to be ongoing and keratin expression patterns are consistent with those found in tonsil epithelial cells in situ (11, 32). These results are summarized in Table 1.
FIG. 2. HC and IF analyses of primary tonsil epithelial cells indicate cellular heterogeneity. Primary tonsillar epithelial cells (days 7 to 10) were grown to confluence on chambered culture slides. Immunostaining was performed with a panel of monoclonal antibodies against epithelial (keratin, involucrin, and ESA) markers and a B-cell marker, CD21. Positive staining is red. For HC, counterstaining was performed with hematoxylin and eosin (blue.) For IF, nuclei are stained with DAPI (4',6'-diamidino-2-phenylindole) (blue). Larger cells (arrows) are negative for ESA (Ber-ep4 clone) and positive for involucrin. This confirms that the phenotypic differences are linked to the differentiation stage.
In vitro infection. If tonsil epithelium is important in EBV pathogenesis, the cells should be infectible with EBV. Coculture with EBfaV-GFP-expressing EBV-producing cells (49), but not incubation with infectious viral supernatant, resulted in infection of all (10 of 10) independent epithelial cultures tested (Fig. 3). This is consistent with earlier findings that cell-free virus is usually incapable of or inefficient at infecting most epithelial cells in vitro (16, 23, 45). The infection frequency with EBfaV-GFP was estimated to be \( \approx 0.01 \) to \( 0.5\% \) of the total cell population by counting GFP-expressing cells with epithelial morphology on day 3 postinfection. To test the possibility that the titer of cell-free virus was too low, we attempted to infect Daudi cells with the cell-free EBfaV-GFP virus. We observed infection levels of up to \( \approx 70\% \) in Daudi cells with the same cell-free virus supernatant that was unsuccessful at infecting primary epithelial cells (Fig. 3C). This indicates that susceptibility to infection, rather than viral titers, is the limiting factor.

To prove that the infected cells were of epithelial origin, they were stained for cytokeratin expression (Fig. 3B and Fig. 4A and B). True infected epithelial cells (Fig. 3A and B) could be distinguished from occasional adherent EBV producer lym-

### TABLE 1. Summary of phenotypic characterization of primary epithelial cultures from the palatine tonsil

<table>
<thead>
<tr>
<th>Marker</th>
<th>Range of expression levels(^a)</th>
<th>Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Keratins</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pankeratins</td>
<td>(+/++)</td>
<td>All</td>
</tr>
<tr>
<td>Keratin 19</td>
<td>(+/++)</td>
<td>Subset</td>
</tr>
<tr>
<td>Keratin 8/18</td>
<td>(+)</td>
<td>Subset</td>
</tr>
<tr>
<td>Keratin 13</td>
<td>(+)</td>
<td>Subset</td>
</tr>
<tr>
<td>Keratin 10</td>
<td>(\sim)</td>
<td>None</td>
</tr>
<tr>
<td>Keratin 8</td>
<td>(+)</td>
<td>Subset</td>
</tr>
<tr>
<td>Keratin 18</td>
<td>(+)</td>
<td>Subset</td>
</tr>
<tr>
<td>Other</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ESA</td>
<td>(\sim)</td>
<td>Majority</td>
</tr>
<tr>
<td>Involucrin</td>
<td>(+/++)</td>
<td>Subset</td>
</tr>
<tr>
<td>Fillagrin</td>
<td>(\sim)</td>
<td>None</td>
</tr>
<tr>
<td>CD21</td>
<td>(\sim)</td>
<td>None</td>
</tr>
</tbody>
</table>

\(^a\) +, clearly positive staining; ++, strong positive staining; \(\sim\), no staining. Variable expression of specific keratins in subsets of cells indicates a heterogeneous population of epithelial cells derived from tonsil crypt and surface epithelium.

FIG. 3. Primary tonsillar epithelial cells are susceptible to in vitro infection with EBfaV-GFP by cell-cell contact but not with cell-free virus. (A) GFP expression could be detected in epithelial cells as early as 24 h postinfection (white arrows). Occasional adherent producer B cells are indicated with white arrowheads. Left panels, bright field; right panels, fluorescence for the same field of cells. (B) GFP-expressing cells are cytokeratin positive (indicated by white arrows). Left panels, red fluorescence for cytokeratins; right panels, green fluorescence for GFP expression on the same field of cells. (C) Infection by cell-free virus was never observed with epithelial cells, but Daudi cells could be efficiently infected (36 h postinfection) with EBfaV-GFP viral supernatant; the infection efficiency was dose dependent.
phoid cells (Fig. 3A and B) based on their extremely bright GFP levels, morphology, and lack of keratin expression. Dual imaging for cytokeratins and involucrin suggests that both undifferentiated and large differentiated epithelial cells could be infected (Fig. 4A to C).

We next tested whether latent viral proteins are expressed and if susceptibility to infection is perhaps limited to the artificial EBfaV-GFP virus strain, which lacks LMP2 (49). We cocultured the epithelial cells with phorbol myristate acetate-butryic acid-stimulated B-95.8 marmoset and IgG-stimulated Akata (not shown) cell lines and performed IF staining for cytokeratins, EBNA1, EBNA2, LMP1, and LMP2. Both virus strains were able to infect epithelial cells, as judged by the detection of EBNA1 and LMP1 protein expression within the same cells (Fig. 4D to I). EBNA2 and LMP2 expression was not detected. These results indicate that latent genes are expressed in the EBV-infected primary tonsil epithelial cells and that infection is not strain specific.

**Ex vivo infection.** During the course of characterization and infection of the primary epithelial cells, we observed that EBV DNA was routinely detectable in the cultures prior to infection (data not shown). Since we employed highly sensitive PCR to detect the viral DNA, we could not distinguish whether the signal was derived from infected cells or from virions, virus fragments, or cell debris from infected cells released by the tonsil explants. To distinguish between these possibilities, we performed RT-PCR analysis for a range of viral genes, including EBNA1U-K, EBNA1Q-K, EBNA2, LMP1, and LMP2 genes. To our surprise, RNA for LMP1 and only LMP1 was robustly and routinely detected in the cultures from 21 of 30 EBV-positive tonsils but in 0 out of 15 cultures from EBV-negative tonsils. Limiting dilutions of Southern-blotted LMP1 RT-PCR products (Fig. 5) illustrate that transcript levels in $2 \times 10^{3}$ to $1 \times 10^{6}$ epithelial cells were equivalent to 10 to 100 control EBV-infected B4 B cells. By comparison, signals for the other genes were detected in only one or two cultures at or close to the detection limit, casting doubt on the significance of these findings. LMP1 is expressed both in latently infected cells and in cells replicating the virus. However, the replicative form is truncated and lacks exon 1. The PCR primers used were designed to bridge exons 1 and 2 and therefore detect only the latent form of LMP1 (53). The reproducible detection of the latent form of LMP1 mRNA demonstrated that the primary cultures contained latently infected cells.

It is unlikely that the LMP1 transcript signals we detected were from viable infected B cells because the cultures were

![Fig. 4](http://jvi.asm.org/)
washed frequently with medium to remove nonadherent cells and the epithelial cells were split twice before analysis by gently washing them with trypsin prior to transfer to 100-mm-diameter dishes. By that time, no B cells could be detected in the cultures by histochemistry or flow cytometry. Moreover, the tissue culture conditions were highly selective against lymphocytes and other nonepithelial cells. Control experiments with primary B cells or EBV-transformed lymphoblastoid cell lines showed that all the B cells were dead after 2 to 3 days of culture in the selective medium (data not shown). This raised the possibility that there were latently infected epithelial cells in the cultures. To identify which type of cell was infected, we performed immunochemistry and fluorescence staining to detect LMP1 protein expression (Fig. 6). We chose LMP1 because the gene was the only one that we found to be reproducibly expressed at the transcript level in the cultures. In control experiments, we were able to reliably detect a single EBV-positive LMP1-expressing cell in a background of $10^5$ EBV-negative cells, indicating the sensitivity of this staining (data not shown). In double-blind studies, we detected LMP1 protein expression in single cells with clear epithelial-cell morphology from 5 of 19 independent 7- to 10-day-old primary epithelial cultures. Subsequent analysis revealed that all five

FIG. 5. RT-PCR for LMP1 expression in primary ex vivo epithelial cells from the palatine tonsil. RT-PCR was performed on RNA from $2 \times 10^3$ to $1 \times 10^6$ day 10 to 14 epithelial cells. The PCR products were fractionated on agarose gels, Southern blotted, and hybridized with an LMP1-specific probe. The size of the PCR product (150 bp) is indicated. Limiting dilution was performed with serially diluted EBV-positive IB4 cells as a positive control (+). EBV-negative BJAB cells and H2O were negative controls (−). Epithelial cultures of 15 individual tonsils are shown; 12 were from EBV-positive donors (T), 9 of which were positive for LMP1, and 3 were from EBV-negative donors (T*), none of which were positive for LMP1.

FIG. 6. LMP1 protein expression in ex vivo tonsillar epithelial cells. Epithelial cells were cultured as described in Materials and Methods and stained between days 7 and 10 with the anti-LMP1 monoclonal (S12) primary antibody for HC or IF. (A) LMP1 expression was assessed in the EBV-positive control B-cell line IB4. (B) Epithelial-cell culture from an EBV-negative tonsil. (C and D) Epithelial-cell cultures from EBV-positive tonsils. Left panel, low power; right panel, high power. (E and F) Epithelial-cell cultures from EBV-positive tonsils analyzed by HC (E) or IF (F). (G and H) Epithelial-cell cultures from EBV-negative tonsils infected in vitro and analyzed by HC (G) or IF (H). Note the similarity in staining observed when LMP1-expressing epithelial cells from an EBV-positive tonsil (ex vivo [E and F]) are compared with epithelial cells from an EBV-negative donor infected in vitro (in vitro infection [G and H]). Panel F shows a typical epithelial perinuclear distribution of LMP1 in an ex vivo-infected cell.
also expressed LMP1 RNA, based on RT-PCR for latent LMP1 transcripts, and were derived from EBV-positive tonsils, based on DNA PCR (Fig. 6C to H), whereas 10 LMP1-negative cultures were derived from EBV-negative tonsils (Fig. 6B). Thus, LMP1-expressing epithelial cells were found in cultures from 5 of 9 EBV-positive tonsils and 0 of 10 EBV-negative tonsils. The level of infection based on LMP1 protein staining was \(~\text{5 to 10 per } 10^6\) epithelial cells (0.005 to 0.01\%), quite similar to the levels of infection reported for the B-cell compartment (27). LMP1 (latent) transcripts were detected in all the cultures where LMP1 protein was found. We were unable to find evidence of EBNA1, EBNA2, or LMP2 expression in any epithelial cells. The absence of EBNA1 may imply that the infected cells were not proliferating (12), and this would be consistent with the observation that the LMP1-positive cells were always found alone, never in clusters. However, as with all histochemical and immunofluorescence studies, it is impossible to resolve whether the failure to find a protein represents absence of the protein or whether the levels of expression were too low to be detected.

One possible explanation for the presence of these infected epithelial cells comes from the well-known phenomenon that B cells latently infected with EBV will replicate the virus when placed in culture. It was conceivable, therefore, that the epithelial cells became infected through cell-to-cell contact with such cells. This interpretation is unlikely, because the explanted cultures were continuously maintained in the presence of a high concentration of acyclovir (0.1 mM) in the media. Acyclovir is a common antiviral agent (29) that effectively blocks viral replication with a 50% effective dose of 0.3 \(\mu\)M, based on blocking viral replication in a lymphoblastoid cell line in vitro. We conclude, therefore, that there are bona fide LMP1-positive, latently infected epithelial cells in the cultures.

Viral replication is a frequently reported consequence of EBV infection of epithelial cells in culture which might be differentiation dependent (10, 22, 23, 28, 47, 54). Since our cultures were routinely grown in the presence of acyclovir, it would not be possible to detect the presence of viral replication. Therefore, we derived epithelial cell cultures in which acyclovir was removed after day 4. The cells were further incubated for 4 to 6 days in the absence of acyclovir in selective media. The epithelial cells were then stained with a cocktail of antibodies recognizing immediate-early (BZLF1), early (EAD), and late (VCA) viral lytic proteins. Lytic antigen expression was detected in two of two cultures from EBV-positive tonsils but in zero of two cultures from EBV-negative tonsils (Fig. 7A to I). Most of the lytic staining was nuclear, indicating EAD or Z protein expression, but occasional cytoplasmic staining (VCA) was also observed (Fig. 7B and C). The cells expressing lytic antigens were usually found in clusters, suggesting that the virus may have spread in the cultures through cell-to-cell contact between epithelial cells. This is a different pattern from what we observed with LMP1, which was always detected only in single cells. This indicates that different cells were expressing LMP1 and the lytic antigens. These data show that cells in primary tonsil epithelial cultures from healthy EBV-positive individuals probably sustain both latent and lytic EBV infection.

**DISCUSSION**

The results presented in this paper relate to three important areas of EBV interaction with epithelial cells. These are: the establishment of a representative primary culture system for a hypothesized target of infection in vivo (tonsil epithelium), the demonstration that a small subset of such cells is infectible in vitro by exogenous virus via cell-cell contact, and the observation that, without adding any exogenous virus, EBV-infected epithelial cells are already present in cultures from EBV-positive individuals.

Tonsil epithelium is a heterogeneous lymphoid organ containing two different types of actively differentiating epithelia, the lining stratified squamous epithelium and the reticulated crypt, or lymphoepithelium. The tonsil crypts represent a specialized compartment, important in the immunological functions of the tonsil because of the immediate proximity of the epithelial and lymphoid tissues (38). Human immunodeficiency virus has been reported to specifically target this region, possibly because of the close interaction of human immunodeficiency virus targets (15). Keratin expression patterns confirmed that our procedure generated heterogeneous epithelial-cell cultures. K8, K18, and K19 expression in subsets of cells suggests that they were derived from the reticular crypt epithelium, and the appearance of large differentiated cells was consistent with an ongoing active process of differentiation in the cultures (11, 32). It is possible that the patterns of keratin expression we observed were induced in vitro. However, primary oral epithelial cells possess intrinsically determined programs for keratin expression, which are believed to be unaffected by short-term in vitro culture (30). Interestingly, early removal of the tissue explants abolished the growth and viability of the remaining adherent epithelial cells. It is conceivable that culturing epithelial cells from tissue explants provides a desirable site-specific mesenchymal influence in initial stages of culture. Mesenchymal cells are absent from cultures in which epithelial cells are isolated from enzymatically digested tissues. This culture procedure often requires an artificial feeder layer, which might benefit the growth of particular subpopulations of epithelial cells (24, 31). Our culture dynamics support the notion that primary epithelial cells are sensitive to their milieu (i.e., stroma), which is likely responsible for the contextual specificity of various epithelia (37, 40).

We consistently detected EBV DNA, RNA, and proteins in the cultures. The question arises as to the origin of these infected cells. The obvious explanation is that we were detecting contaminating infected B lymphocytes. There are several lines of evidence that eliminate this possibility. The level of infected B cells in the tonsil is \(~1 \text{in } 10^5\) (27), so to have a single infected cell would require the presence of \(~10^2\) uninfected lymphocytes, which we would readily detect in the cultures. Furthermore, the selective medium used in the cultures kills all lymphocytes, including EBV-infected B cells, within a few days. Most compelling, though, was the fact that the cells staining positive for LMP1 and lytic proteins had the typical morphology of epithelial cells. Indeed, the elimination of all the lymphocytes, including EBV-infected B cells, may have been important in increasing our chances of detecting the rare infected epithelial cells. Where and how were these cells infected? The only possibility is through contact with infected B
cells in the explants, since the epithelial cells cannot be infected by cell-free virus. Infected B cells in the explant would have survived for only a day or two in the selective medium. Therefore, the epithelial cells would have to have become infected before or shortly after they began migrating onto the tissue culture dish. Since this occurred in the presence of acyclovir, we must conclude that these B cells were already producing infectious virus at the time of explantation. This in turn leads to the inevitable conclusion that the epithelial cells were infected by virus-producing B cells that were already replicating EBV in the tonsil. Alternatively, the epithelial cells were already infected at the time of explantation. It is unlikely that we will ever be able to distinguish between these two possibilities, because the number of infected epithelial cells is so small that it would be impossible to convincingly detect them on a consistent basis in the presence of all of the lymphocytes present when staining cross sections of intact tonsil tissue. Mechanistically, however, the two explanations both lead to the same conclusion, namely, that normal tonsil epithelium is infectible with EBV in situ (whether it occurred in vivo or in vitro), and support the proposed dual tissue tropism of EBV for B and epithelial cells of the crypt regions of the tonsil (8, 37, 59).

FIG. 7. (A to I) Immunofluorescence images of lytic gene expression in primary cultures from two tonsils. Cells were cultured in the presence of acyclovir until day 4. On day 5, SFM plus KGF was added, and the cells were transferred on days 7 and 8 to culture slides. Cytospins of 8- to 16-h IgG-treated Akata cells were used as positive controls (J to M). VCA staining is cytoplasmic (J), whereas Zebra (Z) and early antigen (Ead) staining is nuclear (K and L). Epithelial cells were fixed and stained on day 10 with the pooled monoclonal antibodies against Ead, Z, and VCA. The antibodies were pooled to increase sensitivity (M). The white arrows in panels B and C point to cytoplasmic VCA staining. Merged images are presented to emphasize the predominantly nuclear nature of the positive staining in both cultures derived from Z and Ead (D to L).
The presence in our cultures of both latently and lytically infected cells is fully consistent with their expected properties based on the behavior of EBV in the major associated epithelial pathologies, NPC and OHL, and other previous reports of EBV-infected epithelial cells in vitro (10, 22, 47). Whether our experiments are indicative of a role that epithelium may play in EBV persistence remains unresolved. It might be important to note that the frequency of EBV-infected cells in the primary cultures was similar to that found in B cells from the same tonsils (−0.01%). An average tonsil has a total epithelial surface area of 340 cm² (37). This would represent ~5 × 10⁷ tonsil epithelial cells in vivo (based on the confluency of epithelial cells in a 100-mm-diameter dish), which translates into ~5,000 infected epithelial cells per tonsil at any given time. This is comparable to the estimates of the total number of infected B cells in the tonsils, which is ~10,000 to 50,000 (27).

If epithelial cells do play a role in viral persistence, the failure to infect them with cell-free virus suggests they are not the target of primary infection. Their ability to be infected through cell-to-cell contact means that they could mediate the release of EBV into saliva from infected B cells in the lymphoepithelium. The ready ability of epithelial cells to replicate EBV, combined with the observations that persistent infection is not sustainable in the absence of B cells (17), further supports the idea that the primary role of epithelial cells is to facilitate the release of EBV, not to maintain persistent infection, which is presumably the function of the B-cell compartment. What, then, is the role of the latent infection we have observed? If epithelial cells cannot sustain a persistent infection, we must conclude that latent infection is transient and either an epiphenomenon of the ability of epithelial cells to be infected or possibly a mechanism for short-term persistence, which might provide a more stable source of cells producing infectious virus.

Virion infection of polarized epithelial cells has been reported, but only through the basolateral surface (54). It will be necessary to discover conditions under which our primary tonsillar epithelial cells can be infected at the apical (mucosal) surface with cell-free virus before we can speculate that the epithelial-cell layer may also have a role as the first target tissue in primary EBV infection.

There have been an ever-growing number of reports of successful infection of epithelial cells in vitro. Usually, these studies have employed established cell lines (10, 14, 36, 22, 23, 28, 45–47, 49, 54, 60). Attempts to infect primary nasopharyngeal, ectocervical, and gastric epithelia have been described, with variable success. Nasopharyngeal tissue was reported to be uninfected (16); the ectocervix was reported to be infectible only by oropharyngeal strains of EBV, not laboratory isolates (46); and the gastric epithelium was reported to be infectible in only 3 of 21 attempts (36). We believe, therefore, that this represents the first report of a technique to reproducibly infect representative primary cultures from tonsil epithelium. Reports of viral-gene expression patterns in infected epithelium have also varied. For example, EBV-infected lung, colon, and primary gastric epithelial cells probably do not express LMP1 upon EBV infection (10, 36, 46), but in other reports, EBV-infected epithelial cells of naso- and oropharyngeal origin do express LMP1 (10, 54). These discrepancies may in part reflect technical issues but may also arise because epithelia from different sites are heterogeneous and biologically distinct. We think, therefore, that it is important to study EBV infection in the correct epithelial-tissue background. This concept is reinforced by studies of EBV in B cells, where it is clear that the pattern of viral genes expressed is dependent upon the type and location of the infected cell (5). It was striking that the only latent gene we found reproducibly in the ex vivo-infected cells was the viral oncogene LMP1. At this point, it would be premature to suggest that this reflects a novel form of EBV gene expression, i.e., LMP1 only. This is because the lower limit of the detection sensitivity for the RT-PCR and immunohistochemistry assays for the other viral latent genes is not known and the infected cells are so rare. This makes a negative result difficult to interpret with confidence. Nevertheless, the presence of LMP1 suggests that it is automatically expressed upon EBV infection of normal nasopharyngeal epithelium. Such expression of the oncogenic LMP1 could be the initiating event that may ultimately lead to development of nasopharyngeal carcinomas.

In vitro infection of our primary tonsil epithelial cultures required cell-cell contact, and only a small subset of cells was susceptible to infection. These observations are consistent with the findings of others using different epithelial-cell types (10, 23, 54). There is increasing evidence that viruses exhibit a high degree of specificity for their targets (7). It would be interesting to see what specific characteristics the infectible cells might have and if expression of particular viral-gene transcription programs relates to the differentiation stage of the cells. Evidence for infection specificity in epithelial cells has recently been shown in vitro and might depend on the expression of β1 integrin (54), which is likely to correlate with the differentiation stage (54, 57). Our primary in vitro model with actively differentiating cells might prove a valuable tool to address these issues in the future.

Early findings of EBV in oropharyngeal epithelial cells fueled the notion that epithelium might play a role in viral persistence (1, 45–47). However, subsequent reports were unable to reproduce these findings (25, 35), casting doubt on the role of epithelial cells. Recent findings in culture defining an epithelial-cell receptor (8, 54) for the virus and suggesting a mechanism to explain the potential dual tissue tropism of the virus for B cells and epithelial cells (8, 54) have reopened the issue. However, what is lacking is convincing evidence of infection in vivo. It has not been possible to detect EBV-infected epithelial cells in vivo using EBER hybridization in combination with B-cell and epithelial-cell-specific markers on cross sections of tonsils and in exfoliated epithelial cells in throat washes of infectious-mononucleosis patients (25, 35). This approach was based on the assumption that during infectious mononucleosis there should be a massive amount of epithelial-cell infection that matches the levels seen in B cells, where as many as 50% of the memory cells may be infected (21). From in vitro studies, it is well known that most, if not all, B cells are infectible with EBV; however, the in vitro experiments shown here and by others (54) suggest that only a small fraction of tonsil epithelial cells are infectible with EBV. Therefore, massive infection of epithelial cells may not be possible in vivo during infectious mononucleosis because, unlike B cells, most epithelial cells are not targets of infection. This could explain the failure to detect
infected epithelial cells in the tonsils of acute infectious mononucleosis patients by using histochemical techniques. Our findings support the developing model in which EBV is able to infect healthy epithelium as part of its life cycle (8, 39, 54, 55) and contribute to our understanding of the evolution of naso- and oropharyngeal epithelial pathologies.

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