Feederle R, Neuhierl B, Bannert H et al.Epstein-Barr virus B95.8 produced in 293 cells shows marked tropism for differentiated primary epithelial cells and reveals interindividual...

ARTICLE *in* INTERNATIONAL JOURNAL OF CANCER · AUGUST 2007

Impact Factor: 5.09 · DOI: 10.1002/ijc.22727 · Source: PubMed

6 AUTHORS, INCLUDING:

Claire Shannon-Lowe
University of Birmingham
28 PUBLICATIONS **685** CITATIONS

SEE PROFILE

Int. J. Cancer: **121,** 588–594 (2007)

Epstein–Barr virus B95.8 produced in 293 cells shows marked tropism for differentiated primary epithelial cells and reveals interindividual variation in susceptibility to viral infection

Regina Feederle[1], Bernhard Neuhierl[1], Helmut Bannert[1], Karsten Geletneky[2], Claire Shannon-Lowe[3] and Henri-Jacques Delecluse[1]*

[1]*Department of Virus Associated Tumours, German Cancer Research Center, Heidelberg, Germany*
[2]*Department of Neurosurgery, University of Heidelberg, Heidelberg, Germany*
[3]*Cancer Research UK Institute for Cancer Studies, University of Birmingham, Birmingham, United Kingdom*

Epstein-Barr virus (EBV), a well-characterised B-lymphotropic agent is aetiologically linked to B cell lymphoproliferations, but the spectrum of diseases the virus causes also includes oral hairy leukoplakia, a benign epithelial lesion, as well as carcinomas of the nasopharynx and of the stomach. However, it is still unclear how EBV accesses and transforms primary epithelial cells. Sixteen samples consisting of primary epithelial cells from the sphenoidal sinus or from tonsils were infected with GFP-tagged recombinant B95.8 EBVs produced in the 293 cell line. The rate of infection was assessed by counting GFP-positive cells and cells expressing viral proteins. Primary epithelial cells from all samples were found to be sensitive to EBV infection but there was a marked interindividual variation among the tested samples (2–48% positive cells). This suggests heterogeneity in terms of sensitivity to EBV infection *in vivo* and therefore possibly to EBV-associated diseases of the epithelium. The virus showed a preferential tropism for differentiated epithelial cells (p63 negative, involucrin positive). In all cases, infected cells expressed EBV lytic proteins but also the LMP1 protein. The viral tropism for differentiated cells and the permissivity of these cells for virus replication reproduced *in vitro* cardinal features of oral hairy leukoplakia. We have identified a source of EBV that shows unusually strong epitheliotropism for primary epithelial cells that will allow detailed analysis of virus-cell interactions during virus infection, replication and virus-mediated transformation.

Key words: EBV; epithelial cell infection; OHL; lytic replication

Epstein-Barr virus (EBV) is an ubiquitous human herpesvirus whose infection is usually clinically silent or manifests as a rhinopharyngitis during early childhood.[1] If infection is delayed until adolescence, it can cause an infectious mononucleosis (IM) syndrome associated with significant morbidity and mortality.[1] Following primary infection, the virus establishes lifelong persistence as seen with other herpesviruses. Curiously, much more is known about the site of persistence of the virus, the B lymphocytes, than about the target cells of primary infection or virus reactivation and propagation.[1] In this regard, investigation of tissues from IM patients has led to contradictory conclusions as to whether B cells or pharyngeal epithelial cells are the primary target cells.[2] More recent reports favour the idea that intramucosal B cells are the primary site of infection.[3–5] Similar divergence of views exist regarding the site of EBV replication and propagation. A minority of EBV-infected B cells or plasma cells have been observed to initiate lytic replication *in vivo*.[3,5,6] However, evidence for full virus multiplication and propagation in these cells *in vivo* is still lacking. Whether epithelial cells, or even other cell types, support virus multiplication *in vivo* is still a matter of controversial debate.[2,4,7]

Analysis of epidemiological and molecular data accumulated since the discovery of the virus has led the WHO to classify EBV as a tumour virus.[8] EBV causes at least 1% of all human tumours worldwide, including several subtypes of B and T cell lymphomas but also of gastric and nasopharyngeal carcinomas.[1,9] EBV's genome has also been found *in vivo* in oral hairy leukoplakia (OHL), a benign epithelial lesion characterized by virus infection and replication in the differentiated layers of the tongue epithelium, in cells of the tongue epithelium at autopsies or in rare cells of the tonsillar epithelium.[10–13] EBV's infectious spectrum is therefore not limited to B cells but includes epithelial cells and T cells.[1] One possible interpretation of these data is that epithelial cell infection is not observed in healthy carriers and is only restricted to pathological states.

The nature of the epithelial cell-virus interactions is further obscured by the fact that, in contrast to B cells, primary epithelial cells and T cells are nearly refractory to cell-free infection *in vitro*.[12,14–16] Transfection of CD21, one of the cellular ligand of EBV gp350 surface glycoprotein, in epithelial cell lines markedly increases efficiency of infection suggesting that virus binding to target cells is a limiting factor in most epithelial cell lines.[17] In the same vein, Tugizov *et al*. reported increased infection efficiency in epithelial cell subclones selected for their high virus binding levels.[18] We have recently reported that B95.8 viruses produced in the 293 cell line and bound to primary resting B cells can infect epithelial cell lines with a 1000-fold higher efficiency than the same cell-free supernatant.[19] The increased efficiency of this transfer infection results from the formation of a synapse between B cells and epithelial cells that allow virus transfer. Here we show that the same source of virus has the ability to infect *in vitro* cultured primary epithelial cells with high efficiency. We found that EBV possesses a preferential tropism for differentiated epithelial cells and that there is a marked interindividual variability in the susceptibility to epithelial cell infection. We finally found evidence for lytic replication and latent infection in these cells and discuss the consequences of these findings for the understanding of EBV-associated diseases.

Material and methods

Cells

293 cells stably carrying recombinant EBV-wt B95.8 genomes (2089; 293/EBV-wt) have been previously described.[20] HEK-293 is a human embryonic epithelial kidney cell line. Akata is an EBV-positive Burkitt's lymphoma cell line. Raji is an EBV-positive human Burkitt's cell line. GCi is an EBV-immortalised lymphoblastoid cell line (LCL) with 8% of the cell population showing spontaneous lytic replication. Peripheral blood mononuclear cells were isolated from fresh buffy coat by density gradient centrifugation. CD19-positive B cells were isolated using M-450

This article contains supplementary material available via the Internet at http://www.interscience.wiley.com/jpages/0020-7136/suppmat

***Correspondence to:** Department of Virus-Associated Tumours, German Cancer Research Center, Im Neuenheimer Feld 242, 69120 Heidelberg, Germany. Fax: +49-6221-424852. E-mail: h.delecluse@dkfz.de

Received 14 December 2006; Accepted after revision 26 February 2007
DOI 10.1002/ijc.22727
Published online 6 April 2007 in Wiley InterScience (www.interscience.wiley.com).

CD19 (Pan B) Dynabeads (Dynal, Hamburg, Germany). Cells were grown in RPMI 1640 medium supplemented with 10% FCS. Samples of normal sphenoidal sinus cells were obtained during hypophysectomy. The Ethics Committee of the University of Heidelberg approved the study (approval 392/2005). Tonsillar and sphenoidal sinus biopsy samples were chopped into small pieces, using forceps and scalpel, and transferred directly onto 10-cm tissue culture dishes into low calcium (0.09 mM) keratinocyte serum-free medium (keratinocyte-SFM, Invitrogen, Karlsruhe, Germany) supplemented with bovine pituitary extract (50 μg/ml), recombinant epidermal growth factor (5 ng/ml) and 100 U penicillin per ml, 100 μg streptomycin per ml and 1 μg amphotericin per ml (Invitrogen). Cultures were passaged with a split ratio of 1:4 when 60–80% confluence was reached.

Virus preparations and infection of target cells

Viruses were obtained from induced 293/EBV-wt cells. 4×10^5 cells were transfected in 6-well cluster plates with expression plasmids (0.5 μg each/well) encoding the BZLF1 and the BALF4 gene products, using lipid micelles (Invitrogen). Four hours later, the transfection mixture was removed and cells were kept in keratinocyte-SFM supplemented with 5% fetal calf serum. Three days after transfection, virus supernatants were filtered through a 0.45-μm filter and stored at −74°C until use. To determine the numbers of infectious EBV particles in the supernatant from induced cell lines, human Raji B cells were infected and supernatant required to infect 1 Raji B cell was defined as 1 'green Raji unit'.[21] Primary epithelial cells were infected with viral supernatant for 16 hr, after which the supernatant was replaced by fresh medium. Three days after infection, cells were trypsinized, washed in PBS and the percentage of GFP-expressing infected cells were visually counted in a microscope counting chamber under UV-light.

Transfer infection

Resting B cells (1×10^5) were exposed to EBV for 3 hr at 4°C, washed 3 times in PBS and cells were added to 2.5×10^4 epithelial cells that had been seeded the day before on 48-well plates. After 24 hr coculture, B cells were washed away and transfer infection was assayed 48 hr later by counting the percentage of GFP-positive cells in trypsinised cell suspension.

Immunostaining

Three days postinfection, cells grown on 8-well chamber slides (Nunc, Wiesbaden, Germany) were washed with PBS and fixed for 20 min either with pure acetone (for staining against gp350/220) or with 4% paraformaldehyde followed by a permeabilisation step with 0.1% Triton X-100 for 2 min. Slides were stained for 30 min with mouse monoclonal antibodies specific against LMP1 (S12; BD Biosciences, Heidelberg, Germany), gp350/220 (ATCC 72A1 hybridoma), EBNA2 (PE2), cytokeratin (Pan AB-2; Lab Vision, Walldorf, Germany), BZLF1 (BZ.1), p63 (4A4; BD Biosciences), keratin 4 (6B10; Progen, Heidelberg, Germany), keratin 6 (Ks6.KA12; Progen, Heidelberg, Germany), keratin 10 (RKSE 60; Progen, Heidelberg, Germany), keratin 14 (RCK107; Progen, Heidelberg, Germany), or with rat monoclonal antibody against EBNA1 (1H4), washed 3 times in PBS and incubated for 30 min with a Cy^3-conjugated secondary antibody (Jackson IR, Dianova, Hamburg, Germany). Monoclonal mouse anti-β-Tubulin IV antibody (ONS.1A6; Sigma-Aldrich, Munich, Germany) was used as a marker for ciliated cells. After several washes in PBS, slides were embedded with 90% glycerol and immunofluorescence examined using a fluorescence microscope (Leica, Bensheim, Germany). To exactly determine the number of positive cells, infected samples were trypsinised, washed in PBS and individual cells were dried on glass slides. Fixation and staining procedures were the same as described earlier.

RNA isolation and quantitative reverse transcriptase PCR

Total RNA was isolated using Nucleospin RNA extraction kit (Macherey Nagel, Düren, Germany). 400 ng of RNA was transcribed with avian myeloblastosis virus reverse transcriptase (AMV-RT; Roche, Mannheim, Germany) into cDNA, using a mix of primers specific for various EBV transcripts and specific for GAPDH as described.[22] qRT-PCR assays to detect LMP1, LMP2A, BZLF1 mRNAs and YUK-spliced and QUK-spliced EBNA1 latent transcripts or FQUK-spliced EBNA1 lytic transcripts were performed as described.[22] EBV-positive reference lines included Akata-BL for QUK-spliced EBNA1 transcripts and GCi LCL for LMP1, LMP2A, BZLF1 and YUK- and FQUK-spliced EBNA1 transcripts. Uninfected sEC served as negative control. PCR and data analysis was performed, using the universal thermal cycling protocol, on an Applied Biosystem 7300 real-time PCR System and SDS software version 1.2. All samples were run in triplicates, together with primers for the amplification of the human GAPDH gene in combination with a VIC®-labeled probe (Applied Biosystems, Darmstadt, Germany) to normalize for variations in cDNA recovery. Transcription values were normalized for the amount of RNA per cell in test samples and in reference cell lines, as well as for the number of infected cells in the test samples. Values obtained for the test samples were expressed relative to those seen in the positive control cell lines, which were assigned an arbitrary value of 1. BARF1 and EBER transcripts were analyzed by end point RT-PCR, using random hexamer primer, and AMV-RT for cDNA synthesis (Roche) and primer pairs specific for BARF1, EBER and GAPDH for PCR analysis as described elsewhere.[23,24]

Electron microscopy

Three days postinfection cells were washed in PBS and fixed with 2.5% glutaraldehyde in the same buffer for 20 min at 4°C. Samples were postfixed in 2% osmium tetroxide in cacodylate buffer for 2 hr at 4°C, stained with 184 uranyl acetate (0.5%) for 16 hr at 4°C, washed twice in distilled water, dehydrated in ethanol and embedded in Epon 812. Thin sections were examined by using a Zeiss electron microscope.

Western blot analysis

Cellular extracts were prepared by sonification of cells in RIPA buffer containing 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% NP40, 0.5% sodium deoxychylate, 0.1% SDS and protease inhibitors (Sigma-Aldrich, Munich, Germany) and clearing of the lysate by centrifugation. The protein concentration was determined (Roti Nanoquant; Roth, Karlsruhe, Germany) and equal amount of protein extracts (50 μg) were separated on a 12% SDS-PAGE gel. Protein extracts from EBV-positive Raji cells and GCi LCL cells (50, 12, 3, 0.8 μg) were loaded as positive control. After blotting onto a nitrocellulose membrane (Hybond ECL; Amersham Biosciences, Munich, Germany), EBNA2 and actin expression was detected using a rat anti-EBNA2 (1:250; clone R3) and mouse anti-actin antibody (1:2,500; clone ACTN05, Dianova) in combination with a secondary horseradish peroxidase-conjugated rabbit anti-rat antibody (1:500; DAKO, Hamburg, Germany) and goat anti-mouse antibody (1:1,000; Promega, Hannheim, Germany) followed by ECL-mediated detection (Amersham Biosciences).

Results

Establishment of primary epithelial cell cultures

We have expanded primary epithelial cells from the mucosa that covers the sphenoidal sinus (sEC) or tonsils *ex vivo*. Fresh samples were obtained from 16 individuals who underwent hypophysectomy or tonsillectomy. Established cultures could be passaged 8–12 times, after which they became senescent. They exclusively consisted of epithelial cells that homogeneously expressed pan-cytokeratin (Fig. 1). The majority of the cells also expressed keratins 6 and 14 but not keratins 4 and 10 (data not shown) and the cells were negative for CD21-specific mRNA transcripts as assessed by RT-PCR (data not shown). Sphenoidal sinus mucosa contains squamous and ciliated cells. To determine which of these cell types were present in our primary cultures we stained 6 early

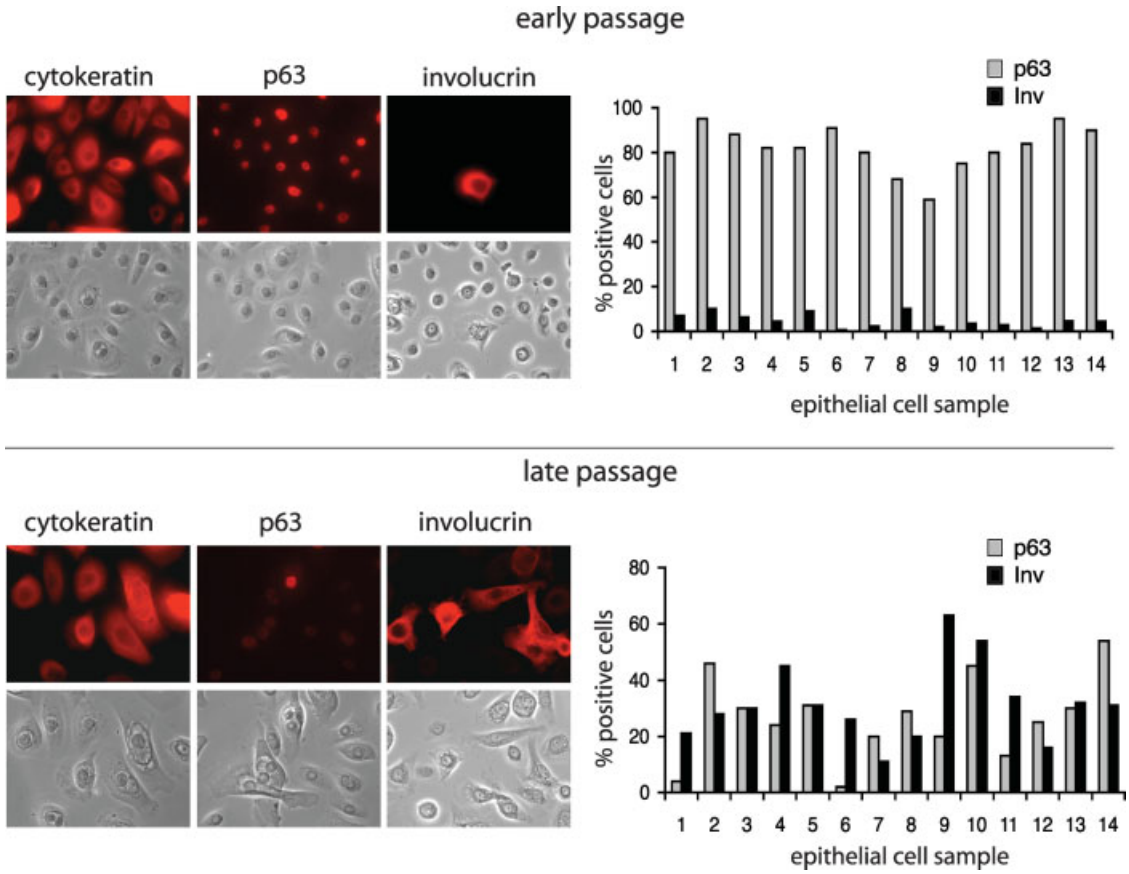

FIGURE 1 – Phenotypic characterisation of primary sEC samples. Cells at early and late passages all stained positive for pan-cytokeratin, confirming their epithelial lineage. Most early passage cells also expressed high levels of p63, a marker of basal cells from stratified squamous epithelia, whereas involucrin expression was restricted to a minority of cells. Late passage epithelial cells showed the reverse pattern of expression with many cells expressing involucrin but only low levels of p63. Immunostainings of sEC sample 9 at early and late passage is shown as an example (×40 magnification).

passge sEC samples with an antibody specific to β-tubulin IV. This antibody strongly reacts with ciliae of ciliated cells and is therefore the marker of choice.[25] One example of immunostaining is shown in Supplementary Figure 1. Ciliated cells isolated from fresh biopsies of the sphenoidal sinus were used as a positive control in this experiment. This assay demonstrated that early passage cultures were devoid of ciliated cells. We then characterised the differentiation state of sEC cultures by staining them with antibodies specific to p63 and involucrin. Basal squamous epithelial cells are strongly p63-positive ($p63^{high}$) and involucrin negative, whereas more differentiated cells are involucrin-positive and express only low levels of p63 ($p63^{low}$).[26,27] At an early passage, the majority of the cells in the sEC cultures were $p63^{high}$ but rarely stained for involucrin (Fig. 1). The expression of p63 provides further evidence for the absence of ciliated cells in our cultures as those do not express p63.[25] In summary, early passage cultures mainly consist of β-tubulin IV-negative p63-positive involucrin-negative epithelial cells, a phenotype characteristic of basal squamous cells. Late passage cultures showed a reversed phenotype with many cells expressing involucrin and being $p63^{low}$ (Fig. 1). Cells were on average also much larger in size than their early passage counterparts. The proportion of $p63^{high}$ and involucrin-positive cells provided a very easy means to distinguish between undifferentiated and differentiated epithelial cell cultures.

EBV produced in 293 cells infects primary epithelial cells

We started by infecting one sEC sample either with cell-free undiluted EBV supernatant (direct infection) or using the recently described transfer infection method with virus bound to resting B cells.[19] Infections were performed with a recombinant EBV (293/EBV-wt) that carries the GFP gene thereby allowing easy identification of infected cells.[20] Infected cultures were assayed for GFP expression and were found to contain 8.5 and 12% positive cells after transfer and direct infection, respectively (Fig. 2). This unexpected result showed that direct infection was not only the easiest but also the most efficient method for studying primary epithelial cell infection and was henceforth used. Control experiments were performed with supernatants obtained from B95.8 marmoset cells that spontaneously replicate. Both transfer and direct infections with B95.8 viruses were consistently negative. We then extended our infection experiments to primary cultures established from 2 normal tonsils. The 2 samples showed infection rates of 13 and 17% by direct infection, thereby excluding that sensitivity to EBV infection is a property unique to sECs (data not shown). In all cases infected cells could not been further passaged.

Interindividual variation in sensitivity of primary epithelial cells to EBV infection

We then extended our infection assays to a total of 14 early passage sEC samples. Initial experiments showed a great variability in the observed infection rates. The differences between cell samples were best visible when infection were performed with diluted supernatants (8 equivalent 'green Raji units' per target cell). Infections were repeated between 3 and 20 times and a representative experiment presented in Figure 3 showed infection rates ranging from 0.1 to 9% in early passage cultures. These results therefore suggest interindividual variability in the sensitivity to EBV infection and possibly to EBV-associated diseases.

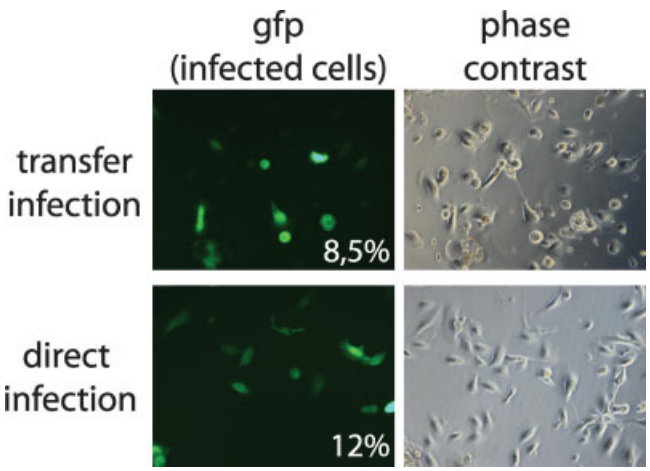

FIGURE 2 – Transfer and direct infection of sEC cells. sEC cells (sEC2 at passage 7) were successfully infected by transfer infection with B cells or using undiluted cell-free viral supernatant as assessed by the identification of GFP-positive cells under UV exposure. The percentage of GFP-positive infected cells 3 days after infection is indicated.

EBV preferentially infects differentiated primary epithelial cells

We then characterized EBV-infected cells within the sEC samples by immunostaining them for p63 and involucrin. More than 90% of EBV-infected GFP-positive cells were involucrin positive. Similarily, p63 stainings showed that about 90% of EBV-infected GFP-positive cells were p63 negative and around 10% were $p63^{low}$ (Fig. 4). These findings demonstrate that EBV has a preferential tropism for nonbasal cells. We further analysed the influence of differentiation on the susceptibility of epithelial cells to EBV infection by comparing infection rates in early and late passage cultures of the same samples. We reasoned that if EBV had a preferential tropism for differentiated cells, late passage cultures should be more susceptible to infection than early passage cultures as the proportion of cells undergoing differentiation in primary epithelial cultures increases with the number of passages. Infection of late passage cultures were performed with the diluted supernatants used for infection of early passage cultures to allow precise comparison between the tested samples. Infection rates were 2–80-fold higher in late than in early passage cultures, with infection rates ranging from 0.2 to 25% (Fig. 3). Here again, the tested samples showed marked interindividual heterogeneity in susceptibility to virus infection.

EBV undergoes lytic replication in infected primary epithelial cells

Next we assessed the fate of viral infection using sEC sample 9 that showed high susceptibility to infection (up to 48% infected cells with undiluted supernatant). We first analysed infected sEC using electron microscopy. Virions undergoing maturation and mature virions were readily visible in infected epithelial cells thereby providing clear evidence for lytic replication (Fig. 5*a*). This finding was supported by the observation that GFP-positive cells produced the immediate early transactivator BZLF1, the early antigen BMRF1 and the late lytic proteins gp110 and gp350/220 as assessed by immunostains (Fig. 5*b* and data not shown). About 90% of GFP-positive cells were found to produce BZLF1 and about half of these cells produced gp350/220 3 days after infection. This suggests that not all infected cells had completed lytic replication by the time of observation and/or that not all infected cells support full lytic replication. These experiments therefore unequivocally identified differentiated epithelial cells as a cellular host for viral lytic replication. We then extended immunostaining assays to the remaining 13 sEC samples and found that

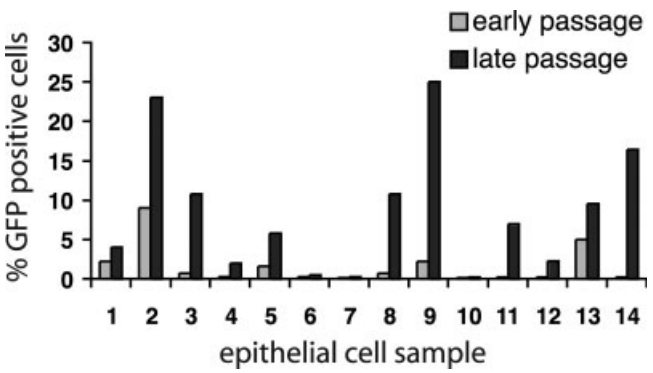

FIGURE 3 – Infection of sEC cell samples at early and late passage. Fourteen sEC samples were infected at early and late passages, and infection rates were estimated by counting GFP-expressing cells on day 3 after infection. Between 0.1 and 9% of the cells were infected in early passage samples (grey bars) and between 0.2 and 25% in late passage samples (black bars). Late passage cultures from the same samples therefore show a 2–80 times higher susceptibility to infection. Infections were performed with 8 'green Raji units' per cell.

all samples expressed lytic proteins thereby confirming our previous results (data not shown).

Infected epithelial cells express EBNA1, LMP1, LMP2A, BARF1 and EBERs

In further experiments we extended the phenotypic analysis of infected sEC by looking at expression of latent EBV genes. Infected sEC produced EBNA1 but not EBNA2 as assessed by immunostaining and western blotting (data not shown and Supplementary Fig. 2). EBNA1 transcripts were mainly initiated from the EBNA1 Qp promoter although some transcripts from the Wp/Cp and Fp promoters were detectable by quantitative RT-PCR (qRT-PCR) (Fig. 5*c*). Further, we could readily detect LMP2A-specific transcripts by qRT-PCR, and BARF1 transcripts and EBER small RNAs by RT-PCR in infected cells (Figs. 5*c* and 5*d*). Nearly all GFP-positive cells were LMP1-positive by immunostaining and by qRT-PCR (Figs. 5*b* and 5*c*).

Discussion

Understanding the contribution of EBV to diseases of the epithelium requires a suitable *in vitro* infection system of normal primary epithelial cells. As a first step in this direction, we have identified a source of EBV with the ability to efficiently infect primary epithelial cells from both the sphenoidal sinus epithelium and the tonsillar epithelium. The virus is therefore able to enter epithelial cells from different tissues and it will be interesting to precisely define the EBV infectious spectrum among the different normal human epithelia. Epithelial cells were susceptible to both direct and B cell-mediated transfer infection and both methods were equally efficient. These results suggest that the virus might use both infectious pathways to target epithelial cells *in vivo*. Transfer infection might be more likely in anatomic regions where epithelial cells and B cells show intimate contact, such as in the tonsils, whereas direct infection might take place in epithelia where B cells are rare, such as in the nasopharynx. In addition, these experiments have highlighted an important difference between primary epithelial cells and epithelial cell lines. Using the same virus source we have previously shown that epithelial cell lines are highly sensitive to transfer infection but not direct infection and we now show that primary epithelial cells are sensitive to both direct and transfer infection. The difference between primary epithelial cells and epithelial cell lines might relate to the ability of the virus to bind to these different cells. Indeed, stable transfection of epithelial cell lines with the EBV receptor CD21 markedly increases efficiency of infection.[17] In addition, epithelial cell line

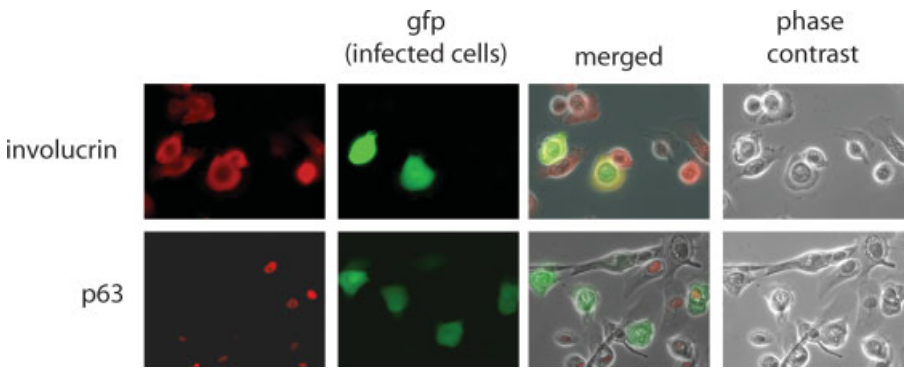

FIGURE 4 – EBV preferentially infects differentiated epithelial cells. sEC cells (sEC9 at late passage) were immunostained for p63 and involucrin 3 days after direct infection. EBV-infected GFP-positive cells were mainly p63 negative but most of them expressed involucrin.

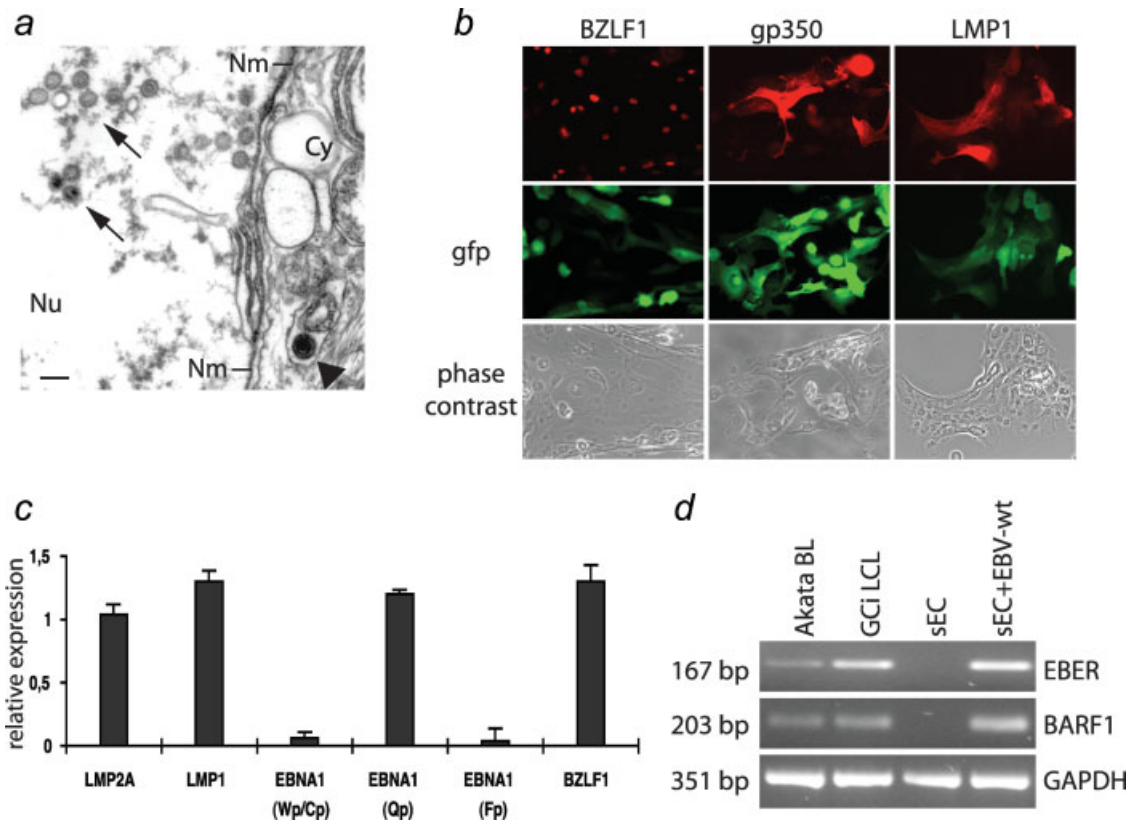

FIGURE 5 – Phenotype of EBV infected epithelial cells. (*a*) Electron micrograph of an EBV infected sEC cell. The nucleus contains immature and mature viral capsids (arrows), mature virions are found in the cytoplasm (arrowhead). Bar: 100 nm; Nu: nucleus; Cy: cytoplasm; Nm: nuclear membrane. (*b*) Immunostains of infected sEC reveal expression of BZLF1 (nuclear staining, ×20), gp350/220 (cytoplasmic staining, ×20) and LMP1 (cytoplasmic staining, ×20). GFP production and phase contrast morphology of infected cells are shown. (*c*) Quantitative RT-PCR analysis was used to quantify viral transcripts in infected sEC. The values given express the ratio between the number of viral RNA molecules measured in infected epithelial cells and in the appropriate positive control lines (Akata-BL for QUK-spliced EBNA1 transcripts, GCi LCL for all other transcripts). Error bars indicate standard deviations. (*d*) EBER and BARF1 expression was assessed by RT-PCR in infected sEC. GCi LCL and Akata cells served as positive controls, uninfected sEC served as negative control. Parallel amplification of GAPDH mRNA was used as internal control for sample loading.

infection rates can be greatly increased by selecting for cells that show strong binding for the virus.[18] This suggests that the majority of cells in an epithelial cell line do not express receptors at levels required for virus binding and could explain why these cells are less susceptible to direct virus infection. Transfer infection, by bringing viruses and epithelial cell lines into contact through a B cell-epithelial cell synapse alleviates the need for direct virus binding and might therefore allow infection of cell lines expressing low levels of epithelial cell receptors. In this regard, assays aiming at detecting GFP-positive cells and EBV-specific mRNAs and proteins have shown that the virus possesses a preferential tropism for involucrin-positive, $p63^{low}$ differentiated epithelial cells. We cannot formally exclude that basal epithelial cells are also susceptible to viral infection but do express neither GFP nor any EBV-specific products. This is, however, very unlikely as the GFP reporter gene is driven from a SV40 promoter that is strongly expressed in most permanently growing cell lines many of them consisting of undifferentiated cells. However, we have demonstrated that EBV gene expression is limited to differentiated squamous cells. The influence of differentiation on infection might result, among other factors, from preferential expression of cellular receptors for EBV on differentiated epi-

thelial cells or by greater stability of the viral genome in these cells.

In our recent paper describing transfer infection of cell lines, we also reported infection of cells from the nasopharynx that could only be infected using transfer infection.[19] This sample, however, behaved very differently than all the samples reported here as infected cells could be grown *in vitro* for 18 passages. In contrast, EBV-infected primary cells from the present study stopped growing and underwent lytic replication. We therefore consider the fresh cell sample from the nasopharynx used in the initial study as an exception.

An important finding of this study is that infection of samples obtained from fourteen different individuals highlighted a great heterogeneity in the sensitivity to EBV infection ranging from 0.2 to 48% among the studied panel. The proportion of differentiated cells varies among the different samples but this fact cannot account entirely for the large variations in infection rates. Indeed many samples, such as sEC sample 9 and 10, or 2 and 3, included very similar proportions of differentiated cells but showed marked differences in the proportion of EBV infected cells (compare Figs. 1 and 3). Whether individuals with higher sensitivity to epithelial EBV infection carry a higher risk for EBV-related epithelial diseases is an interesting possibility but remains to be determined. In this vein, it would be important to determine whether populations at high or medium risk for EBV-associated carcinomas, *e.g.*, from South-East China or Northern Africa have increased sensitivities to epithelial cell infection compared to low risk populations.

Our results might appear to be at odd with previous papers reporting efficiencies of direct EBV infection in primary epithelial cells 2 orders of magnitude lower than the ones we observed (maximum 0.5%).[12,14–16] This might in part result from the requirement of primary epithelial cells to be cultured under conditions favouring differentiation. In addition, these studies might have exclusively analysed primary samples with a low sensitivity to EBV infection. The source of virus used also might have a crucial influence on the ability of the virus to infect its target cells and indeed we could not infect sEC samples with B95.8 viruses produced from marmoset cells. The fact that the majority of laboratories use EBV produced in B cells may provide an explanation for the discrepancies between our and previous reports. It is interesting to note that Sixbey and collegues could infect epithelial cells using throat washings from IM patients but not using viruses produced in cell lines.[28] Using epithelial cell lines infected by a recombinant Akata EBV strain, Borza and Hutt-Fletcher previously reported that viruses produced in B cells infect epithelial cell lines 5 times more efficiently than viruses produced in epithelial cells, reinforcing the idea that the source of virus is important for the outcome of infection.[29] These interesting questions are outside the scope of the present study and further work directly comparing viruses produced in different cell types will be needed to clarify these issues.

The detection of lytic proteins in combination with direct visualization of virions in infected cells confirmed the results obtained by identification of GFP-positive cells and provided strong evidence that EBV undergoes lytic replication in epithelial cells. We have therefore definitely identified primary epithelial cells as a site of EBV lytic replication *in vitro*. These findings add more weight to the suggestion that primary epithelial cells might also be a source of virus *in vivo*, despite the fact that the virus could be identified so far only in rare epithelial cells from tonsils of patients with obstructed-breathing disorders or from the tongue of healthy individuals.[12,13] A common argument against primary epithelial cells being a target of EBV replication is the absence of detectable virus in patients with X-linked agammaglobulinemia who lack mature B cells.[30] We agree that this observation strongly suggests that B cells are the only virus reservoir. However, this does not exclude that epithelial cells could become transiently infected and die after a burst of lytic replication. In fact, IM patients are subjected to clinical investigations several days after onset of the clinical symptoms, which themselves postdate primary infection by several weeks, and by which time infected epithelial cells might have been eliminated from the body.[31]

At this stage of the work the detection of EBNA1, LMP1, LMP2A, BARF1 and EBER transcripts in infected primary epithelial cells either suggests that, in addition to cells undergoing lytic replication, other epithelial cells are latently infected by the virus or that latent proteins are expressed in lytically replicating cells, a situation also observed in OHL (see later). Further work using recombinant viruses devoid of these proteins should help resolving these issues.

Our findings explain many if not all cardinal features of OHL. Both *in vivo* and *in vitro*, EBV shows preferential tropism for differentiated epithelial cells, in which the virus then undergoes lytic replication and expresses, in addition to lytic proteins, some EBV proteins previously found in latently infected B cells. There are, however, some differences regarding the spectrum of latent proteins expressed in primary epithelial cells infected *in vitro*, or in OHL. LMP1 and EBNA1, in particular transcribed from the Fp/Qp promoter, are found to be expressed both *in vivo* in OHL and *in vitro* in our infection system.[32,33] In contrast, the EBNA2 protein was detected in a subset of OHL, but not in primary epithelial cells infected *in vitro*.[32,34] These discrepancies of viral gene expression between epithelial cells infected *in vivo* and *in vitro* might be related to a different microenvironment.

Acknowledgements

We are grateful for helpful discussions with many colleagues during the EBV 2006 meeting at which this work was presented. We thank Mrs. B. Hub for expert technical assistance with the electron microscope, and Ms. E. Kremmer for providing EBNA1 and EBNA2 antibodies. This work was supported by institutional grants.

References

1. Rickinson AB, Kieff E. Epstein-Barr virus. In: Knipe DM, Howley PM, Griffin DE, Lamb RA, Martin MA, Roizman B, Straus SE, eds. Fields virology, 4th edn., vol. 2. Philadelphia: Lippincott Williams & Wilkins, 2001;2575–627.
2. Sixbey JW, Nedrud JG, Raab-Traub N, Hanes RA, Pagano JS. Epstein-Barr virus replication in oropharyngeal epithelial cells. N Engl J Med 1984;310:1225–30.
3. Anagnostopoulos I, Hummel M, Kreschel C, Stein H. Morphology, immunophenotype, and distribution of latently and/or productively Epstein-Barr virus-infected cells in acute infectious mononucleosis: implications for the interindividual infection route of Epstein-Barr virus. Blood 1995;85:744–50.
4. Karajannis MA, Hummel M, Anagnostopoulos I, Stein H. Strict lymphotropism of Epstein-Barr virus during acute infectious mononucleosis in nonimmunocompromised individuals. Blood 1997;89: 2856–62.
5. Niedobitek G, Agathanggelou A, Steven N, Young LS. Epstein-Barr virus (EBV) in infectious mononucleosis: detection of the virus in tonsillar B lymphocytes but not in desquamated oropharyngeal epithelial cells. Mol Pathol 2000;53:37–42.
6. Laichalk LL, Thorley-Lawson DA. Terminal differentiation into plasma cells initiates the replicative cycle of Epstein-Barr virus in vivo. J Virol 2005;79:1296–307.
7. Allday MJ, Crawford DH. Role of epithelium in EBV persistence and pathogenesis of B-cell tumours. Lancet 1988;1:855–7.
8. IARC. Infections with Epstein-Barr virus and human herpes viruses. IARC monographs on the evaluation of carcinogenic risks to humans, vol.70. Lyon: IARC, 1997.
9. Parkin DM. The global health burden of infection-associated cancers in the year 2002. Int J Cancer 2006;118:3030–44.
10. Greenspan JS, Greenspan D, Lennette ET, Abrams DI, Conant MA, Petersen V, Freese UK. Replication of Epstein-Barr virus within the epithelial cells of oral ''hairy'' leukoplakia, an AIDS-associated lesion. N Engl J Med 1985;313:1564–71.
11. Niedobitek G, Young LS, Lau R, Brooks L, Greenspan D, Greenspan JS, Rickinson AB. Epstein-Barr virus infection in oral hairy leukopla-

kia: virus replication in the absence of a detectable latent phase. J Gen Virol 1991;72:3035–46.
12. Pegtel DM, Middeldorp J, Thorley-Lawson DA. Epstein-Barr virus infection in ex vivo tonsil epithelial cell cultures of asymptomatic carriers. J Virol 2004;78:12613–24.
13. Frangou P, Buettner M, Niedobitek G. Epstein-Barr virus (EBV) infection in epithelial cells in vivo: rare detection of EBV replication in tongue mucosa but not in salivary glands. J Infect Dis 2005;191: 238–42.
14. Glaser R, Lang CM, Lee KJ, Schuller DE, Jacobs D, McQuattie C. Attempt to infect nonmalignant nasopharyngeal epithelial cells from humans and squirrel monkeys with Epstein-Barr virus. J Nat Cancer Inst 1980;64:1085–90.
15. Shapiro IM, Volsky DJ. Infection of normal human epithelial cells by Epstein-Barr virus. Science 1983;219:1225–8.
16. Imai S, Nishikawa J, Takada K. Cell-to-cell contact as an efficient mode of Epstein-Barr virus infection of diverse human epithelial cells. J Virol 1998;72:4371–8.
17. Li QX, Young LS, Niedobitek G, Dawson CW, Birkenbach M, Wang F, Rickinson AB. Epstein-Barr virus infection and replication in a human epithelial cell system. Nature 1992;356:347–50.
18. Tugizov SM, Berline JW, Palefsky JM. Epstein-Barr virus infection of polarized tongue and nasopharyngeal epithelial cells. Nat Med 2003;9:307–14.
19. Shannon-Lowe CD, Neuhierl B, Baldwin G, Rickinson AB, Delecluse HJ. Resting B cells as a transfer vehicle for Epstein-Barr virus infection of epithelial cells. Proc Natl Acad Sci USA 2006;103:7065–70.
20. Delecluse HJ, Hilsendegen T, Pich D, Zeidler R, Hammerschmidt W. Propagation and recovery of intact, infectious Epstein-Barr virus from prokaryotic to human cells. Proc Natl Acad Sci USA 1998;95:8245–50.
21. Dirmeier U, Neuhierl B, Kilger E, Reisbach G, Sandberg ML, Hammerschmidt W. Latent membrane protein 1 is critical for efficient growth transformation of human B cells by epstein-barr virus. Cancer Res 2003;63:2982–9.
22. Bell AI, Groves K, Kelly GL, Croom-Carter D, Hui E, Chan AT, Rickinson AB. Analysis of Epstein-Barr virus latent gene expression in endemic Burkitt's lymphoma and nasopharyngeal carcinoma tumour cells by using quantitative real-time PCR assays. J Gen Virol 2006;87:2885–90.
23. Seto E, Yang L, Middeldorp J, Sheen TS, Chen JY, Fukayama M, Eizuru Y, Ooka T, Takada K. Epstein-Barr virus (EBV)-encoded BARF1 gene is expressed in nasopharyngeal carcinoma and EBV-associated gastric carcinoma tissues in the absence of lytic gene expression. J Med Virol 2005;76:82–8.
24. Tierney RJ, Steven N, Young LS, Rickinson AB. Epstein-Barr virus latency in blood mononuclear cells: analysis of viral gene transcription during primary infection and in the carrier state. J Virol 1994; 68:7374–85.
25. Daniely Y, Liao G, Dixon D, Linnoila RI, Lori A, Randell SH, Oren M, Jetten AM. Critical role of p63 in the development of a normal esophageal and tracheobronchial epithelium. Am J Physiol Cell Physiol 2004;287:171–81.
26. Watt FM, Green H. Stratification and terminal differentiation of cultured epidermal cells. Nature 1982;295:434–6.
27. Yang A, Kaghad M, Wang Y, Gillett E, Fleming MD, Dotsch V, Andrews NC, Caput D, McKeon F. p63, a p53 homolog at 3q27-29, encodes multiple products with transactivating, death-inducing, and dominant-negative activities. Mol Cell 1998;2:305–16.
28. Sixbey JW, Vesterinen EH, Nedrud JG, Raab-Traub N, Walton LA, Pagano JS. Replication of Epstein-Barr virus in human epithelial cells infected in vitro. Nature 1983;306:480–3.
29. Borza CM, Hutt-Fletcher LM. Alternate replication in B cells and epithelial cells switches tropism of Epstein-Barr virus. Nat Med 2002; 8:594–9.
30. Faulkner GC, Burrows SR, Khanna R, Moss DJ, Bird AG, Crawford DH. X-Linked agammaglobulinemia patients are not infected with Epstein-Barr virus: implications for the biology of the virus. J Virol 1999;73:1555–64.
31. Svedmyr E, Ernberg I, Seeley J, Weiland O, Masucci G, Tsukuda K, Szigeti R, Masucci MG, Blomogren H, Berthold W. Virologic, immunologic, and clinical observations on a patient during the incubation, acute, and convalescent phases of infectious mononucleosis. Clin Immunol Immunopathol 1984;30:437–50.
32. Webster-Cyriaque J, Raab-Traub N. Transcription of Epstein-Barr virus latent cycle genes in oral hairy leukoplakia. Virology 1998;248: 53–65.
33. Webster-Cyriaque J, Middeldorp J, Raab-Traub N. Hairy leukoplakia: an unusual combination of transforming and permissive Epstein-Barr virus infections. J Virol 2000;74:7610–18.
34. Walling DM, Ling PD, Gordadze AV, Montes-Walters M, Flaitz CM, Nichols CM. Expression of Epstein-Barr virus latent genes in oral epithelium: determinants of the pathogenesis of oral hairy leukoplakia. J Infect Dis 2004;190:396–9.