Epstein–Barr virus replication in tongue epithelial cells

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Epstein–Barr virus (EBV) persistently infects B-cells in humans and can be shed into the saliva. The cellular source of infectious virus is uncertain. Hairy leukoplakia, an AIDS-associated lesion of the tongue, supports EBV replication in epithelial cells. However, the general significance of this observation has remained doubtful. Using immunohistochemistry and in situ hybridization, we demonstrate evidence of EBV replication in tongue epithelial cells in 4 of 168 samples from 84 autopsy cases. Thus, in patients who do not have AIDS, squamous epithelial cells of the tongue rarely support EBV replication. However, all individuals with evidence of EBV replication were either on immunosuppressive therapy or were terminally ill cancer patients, suggesting that an impairment of the immune system may have allowed EBV replication to occur at this site. Thus, our findings are consistent with the idea that EBV replication in oropharyngeal epithelial cells is an infrequent event.

Epstein–Barr virus (EBV) is a ubiquitous herpesvirus which is present in over 90% of the adult human population worldwide, usually as an asymptomatic persistent infection (International Agency for Research on Cancer, 1997). Primary infection commonly occurs during childhood or in early adulthood when it may cause a benign lymphoproliferative disease known as infectious mononucleosis (IM) (International Agency for Research on Cancer, 1997). EBV is able to infect resting human B-cells in vitro converting them into permanently growing lymphoblastoid cell lines (LCL) (International Agency for Research on Cancer, 1997) and establishing a latent infection with expression of a limited set of so-called latent viral proteins (International Agency for Research on Cancer, 1997). Initiated by expression of the BZLF1 protein of EBV, a switch from latent to lytic infection may occur in a small proportion of cells in some LCLs (Miller, 1990; Miller & Lipman, 1973).

In spite of the well-established tropism of EBV for B-cells in vitro, the nature of the cellular compartment(s) mediating primary and persistent infection and virus replication in vivo has been a matter of controversy (Allday & Crawford, 1988; Niedobitek & Young, 1994). In part, this controversy has been resolved by a number of studies pointing to resting memory B-cells as the major site of virus persistence (Niedobitek & Young, 1994; Thorley-Lawson & Babcock, 1999). Latently EBV-infected B-cells are readily detectable in IM and in chronic virus carriers but only exceptional B-cells have been shown to switch from latent to replicative infection in vivo (Niedobitek et al., 1997). Moreover, in these cells usually only BZLF1 has been detected and thus it remains uncertain if these cells support full virus replication with production of infectious virions.

In primary and persistent infection, infectious virus can be shed into the saliva but the cellular source of this virus is uncertain (International Agency for Research on Cancer, 1997). EBV can replicate in squamous epithelial cells of oral hairy leukoplakia (OHL), an AIDS-associated lesion occurring predominantly at the lateral edge of the tongue (Greenspan et al., 1985; Niedobitek et al., 1991; Thomas et al., 1991). However, the relevance of this observation for EBV replication in non-immunocompromised individuals has been questioned (Niedobitek et al., 1991). Using in situ hybridization, the detection of EBV has been reported in desquamated oropharyngeal epithelial cells from patients with IM as well as from healthy chronic virus carriers (Lemon et al., 1977; Sixbey et al., 1984). These results, however, could not be reproduced in two more recent studies (Karajannis et al., 1997; Niedobitek et al., 2000).

In the present study, we wanted to re-examine the potential role of oropharyngeal epithelial cells in EBV replication. In view of the ability of EBV to replicate in epithelial cells of OHL, we hypothesized that if EBV were able to replicate in normal epithelial cells in non-HIV-infected individuals, this should occur at the lateral edge of the tongue, the preferred site of OHL. Since it is virtually impossible to obtain adequate tissue samples from a healthy population, we decided to carry out an autopsy-based study.

Mucosa samples (168) from the left and right lateral margins of the tongue were collected from 84 autopsy cases...
Fig. 1. Immunohistochemistry reveals expression of the BZLF1 immediate early protein of EBV in the nuclei of upper epithelial cells of tongue mucosa (red nuclear labelling) (A). In the same focus, EBV DNA \textit{in situ} hybridization results in an intense labelling of the same cells, suggesting the presence of replicating viral DNA (black grains) (B). In the same sample, immunohistochemistry also reveals expression of EBV VCA (red nuclear labelling) (C) and of EBV MA (red membrane staining) (D). In a different case, \textit{in situ} hybridization shows the presence of large amounts of viral DNA in tongue epithelial cells (E) while there is no immunohistochemical evidence of EBV VCA expression (F). In all cases, immunohistochemistry reveals strong nuclear expression of the proliferation-associated Ki-67 antigen in basal epithelial cells of tongue mucosa indicating antigen preservation (G, red nuclear staining).
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during routine hospital post-mortem examinations carried out at the Institute of Pathology, Friedrich-Alexander-University, Erlangen. Specimens were obtained from 58 males and from 26 females. The age ranged from 40 to 91 years (median 64 years). Malignant tumours were diagnosed in 26 cases. These included carcinomas of the pancreas, prostate, breast, oesophagus, colon, liver and mouth, as well as one case of malignant fibrous histiocytoma. Haematological malignancies were present in six cases. Multiple malignancies were present in four individuals. Fourteen patients had been on immunosuppressive therapy. These included one patient with hepatocellular carcinoma who had received a liver transplant and a patient with non-Hodgkin’s lymphoma who had received a bone marrow transplant. In addition, five individuals had received liver transplants because of liver cirrhosis. There were two heart transplant recipients and one renal allograft recipient. Three patients had received immunosuppressive treatment for autoimmune diseases and one had received treatment with corticosteroids for chronic obstructive lung disease. Finally, a 40-year-old woman had been diagnosed with AIDS. The remaining 47 patients had been diagnosed with cardiovascular disease (34 cases), sepsis (5 cases) and various other diseases (8 cases). Apart from one confirmed AIDS case, there was no clinical evidence for HIV infection in any of our cases.

Samples were fixed in formalin and embedded in paraffin wax according to standard laboratory procedures. Paraffin sections from all 168 tongue mucosa samples were screened for the expression of the EBV BZLF1 protein using immunohistochemistry with the monoclonal antibody (mAb) BZ1 (Dako) (Young et al., 1991). BZLF1 acts as a transactivator of other lytic cycle viral genes and expression of this viral protein is generally taken to be the earliest indicator of lytic EBV replication (Miller, 1990). BZLF1 expression is followed by the expression of early proteins, e.g. BHRF1. Subsequently, viral DNA replication is initiated followed by the expression of late viral proteins, e.g. viral capsid antigen (VCA) and membrane antigen (MA) (Miller, 1990). A total of four samples from three individuals showed a distinct nuclear staining for BZLF1 in the epithelial cells located in the upper layers of the mucosa (Fig. 1A). Thus, the staining pattern was identical to that described previously in OHL (Young et al., 1991). In two cases, BZLF1 expression was observed in one tongue mucosa sample whereas the contralateral sample was negative. In one case, bilateral BZLF1 expression was detected. The BZLF1-positive samples were further analysed for the presence of EBV DNA by in situ hybridization of EBV DNA using 35S-labelled probes as described (Niedobitek & Herbst, 2001). In all four samples positive for BZLF1, this resulted in an intense labelling of nuclei in the same foci showing BZLF1 expression and suggesting the presence of replicating viral DNA (Fig. 1B, E). Expression of two late lytic cycle genes was examined by immunohistochemistry using mAb OT41A, specific for the EBV p40 protein of the VCA complex (BdRF1), and mAb OT6, directed against the virus-encoded MA gp 350/220 (BLLF1) (Hessing et al., 1992; van Grunsven et al., 1993). In three samples, nuclear expression of VCA and membranous expression of MA were also observed in the BZLF1-positive areas (Fig. 1C, D), indicating that in these cases full-scale virus replication with production of infectious virions had occurred. In the fourth sample, viral DNA was detected in addition to BZLF1 expression but VCA and MA were not detectable (Fig. 1F). This might indicate abortive virus replication. Alternatively, it could represent an earlier stage of EBV replication which has not yet proceeded to the expression of late viral proteins. In support of this idea, the focus of replication was much smaller and more superficial in this case. Finally, to detect latent EBV infection, in situ hybridization with 35S-labelled single-stranded RNA probes specific for the small EBV-encoded RNAs, EBER1 and EBER2, was carried out. This resulted in a labelling pattern similar to that seen with EBV DNA in situ hybridization, i.e. nuclear labelling of upper epithelial cells (not shown). This labelling was observed both with antisense and with sense probes and thus was interpreted to reflect hybridization to replicating viral DNA as described previously (Niedobitek et al., 1991). In agreement with previous studies of OHL, no latent EBV infection was detected in epithelial cells outside the foci of replication (Niedobitek et al., 1991). However, the possibility of an EBER-negative form of EBV latency cannot be ruled out by this approach.

Thus we show that squamous epithelial cells at the lateral margin of the tongue can support EBV replication in the same cellular compartment as seen in OHL (Niedobitek et al., 1991) and also in patients who do not have AIDS. Nevertheless, the vast majority of cases in our study lacked immunohistochemically detectable evidence of EBV replication. Although serological data on EBV infection in our cases were not available, an absence of EBV infection is not likely to explain this result. EBV has been shown to infect over 90% of the adult human population worldwide (Crawford & Edwards, 1987). Furthermore, in our hospital, a total of 1667 patient specimens were serologically tested for EBV infection in 2001 resulting in a positivity rate of 90.4% (K. Korn, personal communication). Thus, we assume that the vast majority of our cases would have been EBV-infected. Because we relied on autopsy material, we wanted to rule out antigen degradation as a reason for the negative findings. Paraffin sections from all cases were subjected to immunohistochemical staining for the proliferation-associated Ki-67 antigen, a known labile antigen (Gerdes et al., 1983), using a commercially available antibody (clone MIB1, Dako). This led to a distinct and intense nuclear labelling of cells located in the basal/parabasal cell layer as expected in all cases (Fig. 1G). Thus, antigenic preservation appeared adequate in practically all cases.

Since this was an autopsy-based study, mainly elderly and terminally ill individuals were investigated. Of the three individuals with detectable EBV replication, two had suffered from malignant tumours. One was a 65-year-old male with metastatic pancreatic carcinoma, the other was a 69-year-old...
man with metastatic oesophageal carcinoma. Both patients had received radiochemotherapy. The third case was that of a 49-year-old woman who had received immunosuppressive therapy for antiphospholipid antibody syndrome. In this case, bilateral EBV replication was detected. This raises the possibility that, in these cases, an impairment of the immune system may have allowed EBV replication to occur at this site. In support of this idea, the case showing the most pronounced EBV replication also displayed striking Candida infection of the tongue mucosa, while there was no detectable fungal infection or any other histological abnormality in the other two cases. Interestingly, the only AIDS case included in this study did not show any evidence of EBV replication. Also, patients with other diseases, notably the large group of individuals with cardiovascular disease, did not show evidence of EBV replication. Thus, perhaps the most important finding of this study is the scarcity with which EBV replication occurs in epithelial cells of the tongue even in a cohort of severely ill patients. This observation is in keeping with recent studies and with the idea that EBV replication in oropharyngeal epithelial cells is an infrequent event of uncertain significance for the biology of EBV. However, OHL remains the best studied and apparently most efficient model of EBV replication in vivo (Greenspan et al., 1985; Niedobitek et al., 1991; Thomas et al., 1991; Walling et al., 2001) and, therefore, the role of oropharyngeal epithelial cells for EBV replication in healthy virus carriers deserves further study.

This work was supported by a grant from the Deutsche Forschungsgemeinschaft (SFB466) to G.N.

References


Received 17 December 2001; Accepted 17 July 2002