Detection of lymphocytes productively infected with Epstein–Barr virus in non-neoplastic tonsils

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Epstein–Barr virus (EBV) persists for life in the infected host. Little is known about EBV reactivation and regulation of virus persistence in healthy individuals. We examined tonsils of chronic tonsillitis patients to detect EBV transcripts, EBV genomes and lytic proteins. LMP1 transcripts were observed in 11 of 15 specimens and BZLF1 transcripts were detected in six. Multiple copies of EBV genome equivalents per cell, and ZEBRA- and viral capsid antigen-positive cells were also detected in tonsillar lymphocytes. These results indicate that EBV productively infected cells may survive in the face of immune surveillance in the tonsils. Thus, EBV replication may occur in tonsillar lymphocytes, and tonsillar lymphoid tissues may play a role in the maintenance of EBV load in vivo.

Epstein–Barr virus (EBV) is a human ubiquitous herpesvirus that infects more than 90% of the population worldwide and persists for life in the infected hosts (Rickinson & Kieff, 1996). The patterns of gene expression distinguish distinct states of EBV latent and lytic infection (Kerr et al., 1992). The best characterized state of latent infection is of B cell lymphoblastoid cell lines (LCLs) that have been immortalized by EBV infection in vitro. The latent EBV-carrying LCLs constitutively express the viral genes encoding EBV-encoded nuclear antigens (EBNA) 1–6, latent membrane proteins (LMP) 1, 2A and 2B, two highly expressed RNA species, EBV-encoded small RNAs (EBER1 and EBER2) and BARF0 (Ring et al., 1994; Sadler et al., 1995).

Since there is no in vitro natural permissive system for EBV replication, most studies of lytic virus gene expression have relied on semipermissive EBV-carrying Burkitt’s lymphoma cell lines or LCLs where the virus can be induced to enter the productive cycle by various means, such as exposure to tumour promoters (zur Hausen et al., 1978; Luka et al., 1979), halogenated pyrimidine (Gerber, 1972), antibodies to human immunoglobulin (Tovey et al., 1978) and EBV-encoded transactivator (Countrymann & Miller, 1985). Among the EBV gene products of the replicative cycle, the protein product of the BZLF1 gene, named ZEBRA (BamHI Z fragment, EBV replication activator), acts as the switch from latency to the lytic cycle (Biggin et al., 1987; Carey et al., 1992). The late protein, viral capsid antigen (VCA), encoded by BcLF1 is expressed only in virus-producing cells and is closely associated with the capsid of virus particles (Vroman et al., 1985).

Therefore, expression of both ZEBRA and VCA suggests that EBV-positive cells are undergoing a lytic cycle. Furthermore, DNA–DNA in situ hybridization (ISH) can detect cells productively infected with EBV, since this method can distinguish cells harbouring a low copy number of latent viral DNA from those with multiple copies of replicating viral DNA (Hironaka et al., 1993).

In contrast to the in vitro system, it is still a matter of dispute where the in vivo EBV replication site is. Pharyngeal epithelial cells and oral hairy leukoplakia (OHL) lesions appear to be naturally permissive, with virus replication in differentiating squamous cells leading to the release of infectious virions into buccal fluid (Young et al., 1991; De Souza et al., 1989; Greenspan et al., 1985; Sixbey et al., 1984). Prang et al. (1997) also detected lytic proteins and lytic gene transcripts of EBV in peripheral blood B lymphocytes by immunohistochemistry and RT–PCR in infectious mononucleosis (IM) patients. The result suggests that EBV replication also occurs in lymphocytes. However, it remained unclear where the EBV replication sites are in normal (healthy) individuals. It has recently been shown that the tonsil is one of the candidates for the site of replication of EBV (Babcock et al., 1998; Kobayashi et al., 1998). We have previously found that cells with a high copy number of EBV DNA were located in the upper epithelial cell layers of non-neoplastic tonsil using DNA–DNA ISH and...
Fig. 1. Detection of EBV transcripts. EBER1, BZLF1, EBNA2, LMP1 and LMP2a mRNA were observed in RNA preparations from freshly isolated tonsillar mononuclear cells; 1 µg of total RNA was used as the template for amplification in each case. Control cells were the EBV-positive B95-8 (predominantly latency III, with 5% of cells in the lytic cycle) and EBV-negative Ramos lines, and 1 µg of total RNA was amplified in each case. Histone 3.3 RT–PCR was used to control the quality of the RNA preparations (Prang et al., 1997). dr, Donor; R, right tonsil; L, left tonsil.

**in situ** PCR (Kobayashi et al., 1998). In addition, Babcock et al. (1998) could detect the linear and episomal forms of EBV DNA in tonsillar lymphocytes, while peripheral blood B cells contain only the episomal form. The presence of the linear form may indicate that viral DNA is replicating in the tonsillar lymphoid tissue of healthy, persistently infected individuals. These results suggest that EBV replication occurs in lymphocytes as well as epithelial cells in the tonsillar lymphoid tissue of normal individuals.

In the tonsils, the B cell population makes up 50–70% of total lymphocytes (Yamanaka et al., 1983), much greater than in peripheral blood lymphocytes (PBL), where it is 7–23% (Reichert et al., 1991). The frequency of EBV-infected cells in peripheral blood B lymphocytes was 5–500 per 10^7 (Khan et al., 1996), similar to that in tonsillar cells, 20–100 per 10^7 (Babcock et al., 1998). In addition, there was not much difference in the expression level of EBER1 between the right and left non-neoplastic tonsils of each patient with chronic tonsillitis, although the expression level varied among individuals (Takeuchi et al., 1996), suggesting that the circulating EBV-infected cells were detected in the tonsils from both sides. Therefore, detection of EBV-infected cells may be easier in tonsillar tissues than in PBL. To find EBV replication sites, we examined tonsils by RT–PCR, immunohistochemistry and ISH.

Fifteen tonsillar tissues from eight donors clinically diagnosed with chronic tonsillitis were taken from the Department of Otolaryngology, Kyorin University School of Medicine (Tokyo, Japan). Routine histological analysis showed no involvement of malignant lymphomas or other malignancies. These donors had no history of IM. The antibody titres of the donors to early antigen (EA), VCA and EBNA were examined by EBV-associated antigen detection kits (Kayaku, Tokyo) based on indirect immunofluorescent tests according to the manufacturer’s instructions. Twofold serial dilutions of the serum, from 1:10 to 1:2560, were analysed. These donors, except no. 5, had antibodies to EBNA (data not shown), indicating that they were not in an acute phase of EBV infection. Titres of donor no. 5 (anti-EA, -VCA and -EBNA) were less than 1:10.

To clarify the state of EBV infection in tonsils, RNA from mononuclear cells which were isolated from 15 tonsillar specimens was prepared using TRIzol (Life Technologies) (Ryon, 1999). Then, we tested for expression of EBV transcripts EBER1, BZLF1, EBNA2, LMP1 and LMP2a in these tonsillar mononuclear cells by RT–PCR according to the protocols previously described (Tierney et al., 1994; Chen et al., 1995; Prang et al., 1997). Southern hybridization was performed with locus-specific probes which were labelled with digoxigenin (Roche Diagnostics), after electrophoresis of PCR.

Fig. 2. Detection of EBV productively infected lymphoid cells in the tonsil. (A) HE staining. (B–H) Immunohistochemistry using antibodies to cytokeratin (B), ZEBRA (C; for higher magnification of frame, see D) and VCA (E; for higher magnification of frame, see F); ISH with BamHI W probe (G; for higher magnification of frame, see H). Original magnification × 200 (A, B, C, E and G) and × 600 (D, F and H). These serial sections were obtained from donor no. 3.
Fig. 2. For legend see facing page.
products. EBER1 transcripts were detected in 14 of 15 specimens (Fig. 1). Since EBV-seronegative donor no. 5 did not have these transcripts, the donor may not be infected with EBV. There was little difference in the expression level of EBER1 between the right and left tonsils of each donor as reported previously (Takeuchi et al., 1996). BZLF1 mRNA was also observed in six of 15 specimens. This transcript was detected in both of the tonsils isolated from each donor (nos 2, 3 and 7). However, LMP1, EBNA2 and LMP2a transcripts could not always be detected in both of the tonsils from each individual. Donor no. 3 had all of the transcripts (EBER1, EBNA2, LMP1, LMP2a and BZLF1).

Next, to examine whether the EBV lytic proteins were present in the tonsils, we performed immunohistochemistry. Serial sections of formalin-fixed, paraffin-embedded tonsils were deparaffinized, rehydrated and treated with 0.2% trypsin (DAKO) for antigen retrieval. These sections were reacted with anti-ZEBRA, -VCA and -cytokeratin (marker of epithelial cells, clone AE1/AE3) monoclonal antibodies. Primary antibodies to ZEBRA and cytokeratin were purchased from DAKO. Anti-VCA antibody was obtained from Biogenesis. Immunohistochemical detection demonstrated that ZEBRA and VCA were expressed in 7–10 of 700 tonsillar mononuclear cells with cytoplasmic immunoreactivity in three of eight donors (nos 2, 3 and 7) (Fig. 2C–F). In morphology, these ZEBRA- and VCA-positive cells most resembled lymphocytes (Fig. 2D, F). ZEBRA and VCA were observed in the same area as tonsillar mononuclear cells (Fig. 2C, E). To assess the relationship between the epithelium and EBV lytic proteins, serial sections were reacted with antibodies to ZEBRA, VCA and cytokeratin. In these sections, ZEBRA- and VCA-positive cells were not localized in the epithelium (Fig. 2B, C, E). As a control, no immunoreactivity was obtained with samples lacking primary antibody (data not shown). BZLF1 transcripts could not be detected in donor nos 1, 4, 5, 6 and 8 (Fig. 1), who did not have ZEBRA-positive cells by immunohistochemistry (data not shown). Donor no. 7 has a weak BZLF1 signal compared with the other BZLF1-expressing donors. The number of ZEBRA- and VCA-positive cells in this donor is a little less than that in the other donors as assessed by microscopic observation (data not shown). The frequencies of ZEBRA- and VCA-positive cells depended on the quantity of BZLF1 transcripts.

Furthermore, we analysed the tonsils for the presence and localization of EBV DNA-positive cells. DNA–DNA ISH was performed using a BamHI W probe labelled with alkaline phosphatase. The labelled probes were visualized with nitro blue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate (Life Technologies). BamHI W-positive cells were found to correspond to lymphoid cells. On the other hand, all other tonsillar components including endothelial cells, fibrocytes, granulocytes and macrophages did not have EBV BamHI W fragments (Fig. 2G, H). As a control, no signal was obtained with lack of BamHI W probes (data not shown). EBV DNA-positive cells were localized in the same area as ZEBRA- and VCA-positive cells (Fig. 2C, E, G). However, ZEBRA and VCA were not always expressed in EBV DNA-positive cells. It appears that ISH is more sensitive than the immunohistochemistry. We failed to specify the type of cells which were ZEBRA-, VCA- and EBV DNA-positive by double staining with B-cell-specific antibodies (data not shown). The B-cell-specific antibodies may not react with terminally differentiated plasma cells which were reported to be linear EBV DNA-positive cells by Babcock et al. (1998). Weak signals for EBV-positive cells were also detected in the epithelium in three of seven seropositive donors (nos 2, 3 and 7) (data not shown).

Table 1. Summary of EBV infection in tonsils

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* Analysed by ISH.
† Detected by RT–PCR.
§ Tested by immunohistochemistry.
shown). This seems to be consistent with our previous finding that multiple copies of EBV genomes could be detected in the epithelium of non-neoplastic tonsils (Kobayashi et al., 1998).

RNA–DNA ISH for the detection of EBER1 was also performed using the paraffin sections prepared from the same donors (nos 2, 3 and 7). Although EBER1 could be detected in lymphoid cells, the localization of these cells was different from ZEBRA- and VCA-positive areas (data not shown). This result suggests that EBV-producing cells down-regulated the expression of EBER1 as reported for OHL (Young et al., 1991). Table 1 summarizes the results of Figs 1 and 2.

The CD8+ T-cell population comprised 20–30% (data not shown) of peripheral blood mononuclear cells. By contrast, they accounted only for about 5–8% of tonsillar mononuclear cells as determined by flow cytometry (data not shown). Thus, in the tonsil, the CD8+ T-cell population was smaller than in peripheral blood. EBV-associated antigen-specific CTL lines are CD8+ (Fukukawa et al., 1981; Elliott et al., 1997; Misko et al., 1980). Since EBV-associated antigen-specific CTL cells in tonsils may be fewer than in peripheral blood, EBV-infected cells expressing the EBV-associated antigens targeted by CTL may survive in tonsils. Thus, the tonsil may be a suitable environment for EBV persistence. However, Hirao et al. (1996) found that EBV-specific CTL reactivated from tonsils showed more effective responses to autologous LCL after their first stimulation than those from peripheral blood, but unstimulated tonsillar T-cells had no activity against autologous LCL. These results suggest that EBV-specific CTL activity in the tonsil may be inhibited by certain agents, such as cytokines or unknown agents. Furthermore, LMP1 transcripts were detected in all EBV-seropositive donors, and BZLF1 transcripts were found in three donors (Fig. 1). The transient expression of LMP1 in the tonsillar lymphocytes may indirectly prolong the lifespan of EBV-infected cells, since LMP1 is also known to induce the expression of the cellular oncogene bcl-2 and to prevent apoptosis (Henderson et al., 1991), as suggested previously (Kobayashi et al., 1998). The BZLF1 transcript-positive donors also had ZEBRA- or VCA-expressing cells which localized to the same area of the tonsils. These results are different from PBL studies in which none of the normal individuals gave positive signals for LMP1 and BZLF1 transcripts (Tierney et al., 1994; Chen et al., 1995), and indicate that virus replication might occur in tonsillar lymphocytes.

It is well known that the interleukin 10 (IL-10) coding sequence is highly homologous to the EBV open reading frame BCRF1 (Moore et al., 1990; Vieira et al., 1991). BCRF1 protein, also termed viral IL-10 (vIL-10), inhibits the synthesis of T-helper 1 cytokines (Rousset et al., 1992) and CTL activity (de Waal Malefyt et al., 1991). Therefore, EBV-associated antigen-specific CTL activity might be down-regulated by vIL-10 in EBV-infected areas of the tonsil. Thus, EBV is much more likely to survive in the face of immune surveillance in the tonsils, suggesting that the immune response to EBV in tonsils may be different from that in PBL.

Our results demonstrate that tonsillar lymphocytes are one of the EBV replication sites and a reservoir for EBV in normal individuals.

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