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Role of Epstein-Barr Virus Infection in Oral Diseases

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To determine the role of the Epstein-Barr virus (EBV) in the pathogenesis of oral diseases, 105 formalin-fixed, paraffin-embeded specimens were examined. These specimens included 22 cases of oral squamous cell carcinoma, 21 cases of fibroma, 19 cases of epulis, 16 cases of oral leukoplakia, 10 cases of plemorphic adenoma, 9 cases of oral papilloma and 8 cases of oral lichen planus (OLP). The specimens were analyzed using for polymerase chain reaction (PCR), PCR-Southern blot hybridization, and *in situ* hybridization (ISH) with peptid nucleic acid probes for EBV-encoded small RNAs (EBER) and immediate early mRNAs of the *Bam*H fragment of lower strand frame (BHLF). PCR products for EBV DNA were detected in 68.5% of the examined specimens. In the ISH analysis, EBER and BHLF hybridization signals were mainly localized in the infiltrating lymphocytes. Although the proportion of EBER or BHLF positive lymphocytes varied among the examined diseases, OLP specimens expressed a significantly high level of EBER or BHLF. These results suggest that EBV infection may be implicated in the pathogenesis of OLP.

Introduction

Epstein-Barr virus (EBV), a member of the herpes virus family, is endemic in all human populations. Most people are infected with EBV in early childhood and carry EBV for life, usually without clinical symptoms. If a person is initially infected with EBV during their teenage years, infectious mononucleosis may occur[1]. EBV infection causes human malignancies in both epithelial cells and B lymphocytes. Nasopharyngeal carcinoma, Burkitt's lymphoma and immunoblastic lymphomas in immunosuppressed patients are often associated with EBV infections[2]. Similar associations have recently been shown in benign lesions, including oral hairy leukoplakia of the tongue in immunocompromised hosts[3][4] and Warthin's tumor of the salivary gland[5].

EBV is a B-lymphocytotropic virus[6] and may give rise to either a latent or a productive lytic infection. During latent infections, limited transcription of the viral genome expresses the EBV nuclear antigens (EBNA; EBNA-1, EBNA-2, EBNA-3a, EBNA-3b, EBNA-3c and EBNA-leader protein) and latent membrane proteins (LMP; LMP-1, LMP-2a and LMP-2b)[7]. During productive lytic infections, extensive transcription of the viral genome expresses several viral gene products, including the immediate early antigen, viral capsid antigens and membrane antigens[7]. These products, except for EBNA-1, are selectively attacked and destroyed by cytotoxic T cells.

The detection of EBV DNA within saliva[1] and

a high incidence of EBV DNA detection in tissues near the oropharynx[8)~10)], suggests that oropharyngeal tissues act as a reservoir, leading to latent EBV infections[11)]. However, the potential pathogenic role of EBV in oral diseases has not been clarified. The aim of this study was to investigate the role of EBV in a variety of oral lesions, including benign and malignant tumors as well as non-neoplastic mucosal disorders. EBV can be detected in tissues using with several methods, including polymerase chain reaction (PCR) for EBNA and *in situ* hybridization (ISH) for EBV-encoded small RNAs (EBER).

In the present study, we used the nested PCR[12)], PCR-Southern blot hybridization[12)] and ISH analysis with peptid nucleic acid (PNA) probes for EBER and immediate early mRNAs of the *Bam*H fragment of lower strand frame (BHLF), which is abundent in lytic infections.

Materials and Methods

1. Patients and tissue preparations

This investigation was performed using archival, formalin-fixed, paraffin-embedded specimens from 105 cases obtained during biopsy or surgical procedures performed by the Department of Oral and Maxillofacial Surgery at hospital of Tokyo Women's Medical University. The 105 cases consisted of 22 cases of oral squamous cell carcinoma (OSCC), 21 cases of fibroma, 19 cases of epulis, 16 cases of oral leukoplakia (OL), 10 cases of pleomorphic adenoma (PlA), 9 cases of papilloma and 8 cases of oral lichen planus (OLP).

A nasopharyngeal carcinoma patient with serologically confirmed EBV infection served as a positive control. We have chosen a normal paraffin-embedded liver tissue without EBV infection[8)10)], in which several times of PCR trials proved to be a negative control.

All patients had given their informed consent to participate in the study.

2. Extraction of DNA from paraffin-embedded tissue samples

Multiple 6-μm-thick sections of each sample were cut from the specimens. The sections were deparaffinized with xylene and washed in pure ethanol. After vacuum-drying, the sections were suspended in 500 μl of Tris-HCl EDTA (TE) buffer containing 0.5% sodium dodecyl sulfate (SDS) and 100 μg/ml proteinase K, incubated overnight at 42 ℃, and extracted with phenol, chloroform and isoamyl alcohol. The specimens were centrifiuged at 10,000 rpm for 10 min. Five microliters of 3 M CH_3COONa (pH 5.2) and 50 μl of 2-propanol were then added to the samples, which were allowed to stand at −20 ℃ for 2 hr. After being centrifuged for 20 min at 4 ℃, the DNA samples were washed in 75% ethanol, dried and resuspended in 50 μl of TE buffer.

3. Nested PCR

Two-primer set that amplify a 297 base pair (bp) and a 209 bp fragment of the EBNA-1 gene were used for the nested PCR (Table 1). Consistent DNA amplification result were reproduced in two successive trials.

The total reaction volume was 100 μl, containing 10 μl of Taq buffer, 5 μl of dNTP mixture, 2.5 unit of Taq DNA polymerase (Takara, Otsu, Japan), 500 μg of the DNA sample and 150 pmol of the EB-3 and EB-4 primers in the first round or 300 pmol of the EB-1 and EB-2 primers in the second round. Thirty-five cycles of DNA denaturation (1 min at 95 ℃), annealment (1 min at 55 ℃ for the first round and 60 ℃ for the second round) and elongation (1 min at 72 ℃) were performed in an automated thermal cycler (ATTO, Tokyo, Japan). After the first amplification with the outer primers, 5 μl of the PCR products were transfered from the first to the second reaction mixture. After the second amplification with the inner primers, 5 μl of the PCR products were electrophoresed on an 8% agarose gel containing

Table 1 Sequences of primers and internal probe used in nested PCR and Southern blot hybridization

Primers/Probe	Nucleotide sequences	Base location	Amplified products
Outer : EB 3	5′-AAGGAGGGTGGTTTGGAAAG-3′	109332–109351	297 bp
Outer : EB 4	5′-AGACAATGGACTCCCTTAGC-3′	109609–109628	
Inner : EB 1	5′-ATCGTGGTCAAGGAGGTTCC-3′	109353–109372	209 bp
Inner : EB 2	5′-ACTCAATGGTGTAAGACGAC-3′	109542–109561	
Probe : EBP 5	5′-GTTTAAGAGCTCTCCTGGCTA GGAGTCACGTAGAAAGGA-3′	109400–109439	

0.5 μg/ml ethidium bromide. The gels were photographed under ultraviolet illumination and regarded as positive when a band corresponding to the 209 bp DNA fragment appeared.

4. Southern-blot hybridization

After the nested PCR, the gels were treated with 0.4 N NaOH/1 M NaCl solution to denature the DNA and transfered to a nylon membrane (ATTO, Tokyo, Japan) using the capillary method. The internal probe EBP-5 (Table 1) was end-labelled with a digoxigenin oligonucleotide tailing kit (Roche, Indianapolis, USA) and used for prehybridization and hybridization at 42 ℃ for 4 hr.

After hybridization the membrane was washed twice with a 2× saline sodium citrate (SSC)/0.1% SDS solution for 5 min at room-temperature, twice with a 0.1 × SSC/0.1% SDS solution for 15 min at 42 ℃, and twice with a 0.1 M Tris HCl (pH 7.5) /0.15 M NaCl solution containing 0.2% Triton X for 10 min at room-temperature. The nylon membrane was pretreated with a blocking reagent and incubated overnight at 4 ℃ with an alkaline phosphate-conjugated antidigoxigenin antibody. The membrane was then washed and chemiluminesced with CDP-Star™ (Roche, Boehringer Mannheim, Germany).

The hybridization signals were then detected by exposing the membrane to an X-ray film (Rxu FUJI, Tokyo, Japan).

5. ISH with PNA probes

EBER and BHLF mRNA were detected by ISH using PNA probes and a PNA ISH detection kit (DAKO, Glostrup, Denmark), according to the manufacturer's protocol.

In brief, 3-μm-thick sections on glass slides were deparaffinized in xylene, rehydrated, and digested with proteinase K for 30 min at room-temperature. Then, the sections were treated with 20% acetic acid for 30 seconds at 4 ℃ and dehydrated with pure ethanol. The ISH was performed in a humidity chamber for 90 min at 55 ℃. After hybridization, the sections were subimerged in a stringent wash solution for 25 min at 55 ℃, rinsed in Tris-buffered saline (TBS) at room-temperature, and then incubated for 30 min at room-temperature with an anti-fluorescent isothiocyanate conjugated with alkaline phosphatase.

Visualization was achieved by staining with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate disodium salt for 30 min to 3 hr in the dark and counterstaining with methyl green.

6. Statistical analysis

The statistical significance of the ISH-positive lymphocytes and EBER or BHLF-labelling index was calculated using the Student's t-test. The number of positive lymphocytes present in twenty high-power fields was counted, and the EBER or BHLF-labelling index was calculated as

Table 2 EBV genome expression in the oral diseases examined

Histological diagnosis	No. of cases	PCR DNA positive cases	EBER mRNA positive cases	EBER mRNA LI (%)		BHLF mRNA positive cases	BHLF mRNA LI (%)	
OSCC	22	18	2	9		0	0	
Fibroma	21	11	0	0		3	4	
Epulis	19	16	4	11.7		2	6	
OL	16	8	2	10		0	0	
PIA	10	6	2	5		0	0	
Papilloma	9	6	1	6.8	*	1	6.3	*
OLP	8	7	5	36.8		2	16.4	

*p < 0.001 (paired Student's t-test). OSCC : oral squamous cell carcinoma, OL : oral leukoplakia, PIA : pleomorphic adenoma, OLP : oral lichen planus. LI (%) indicates the mean percentage of EBER or BHLF positive cells in a total number of the lymphocytes.

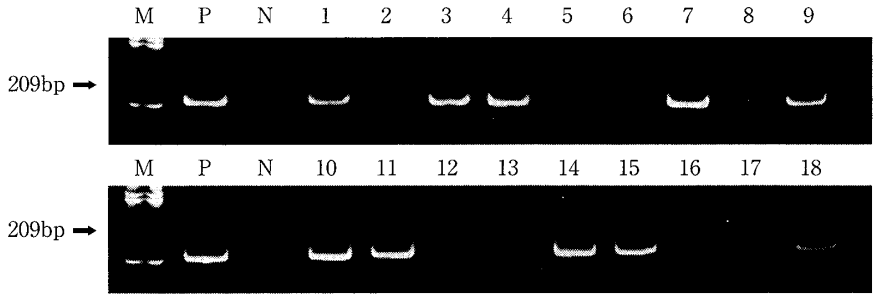

Fig. 1 Gel electrophoresis of nested PCR products for EBV DNA
M: marker, P: positive control, N: negative control, 1~3: oral squamous cell carcinoma, 4~6: fibroma, 7~9: epulis, 10~12: oral leukoplakia, 13~15: pleomorphic adenoma, 16~18: papilloma.

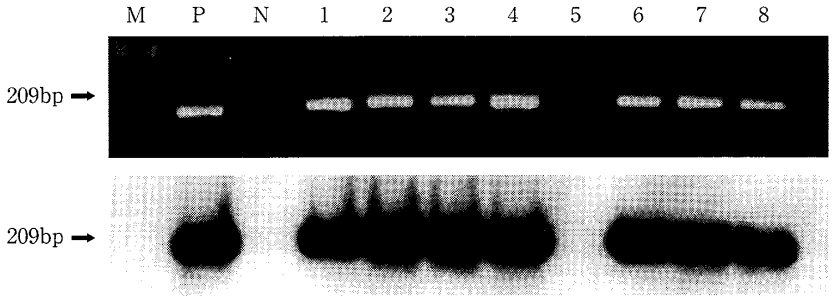

Fig. 2 PCR and PCR-Southern blot hybridization analysis of EBV DNA in oral lichen planus
M: marker, P: positive control, N: negative control, 1~8: oral lichen planus.

the percentage of positive cells out of the total number of lymphocytes for each case.

Results

PCR products for EBV DNA were detected in 68.5% of the examined specimens: 18 of the 22 OSCC specimens, 11 of the 21 fibroma specimens, 16 of the 19 epulis specimens, 8 of the 16 OL specimens, 6 of the 10 PIA specimens, 6 of the 9 oral papilloma specimens, and 7 of the 8 OLP specimens (Table 2, Figs. 1 and 2).

ISH signals were detected in 24 of the specimens that had positive PCR-results for EBV

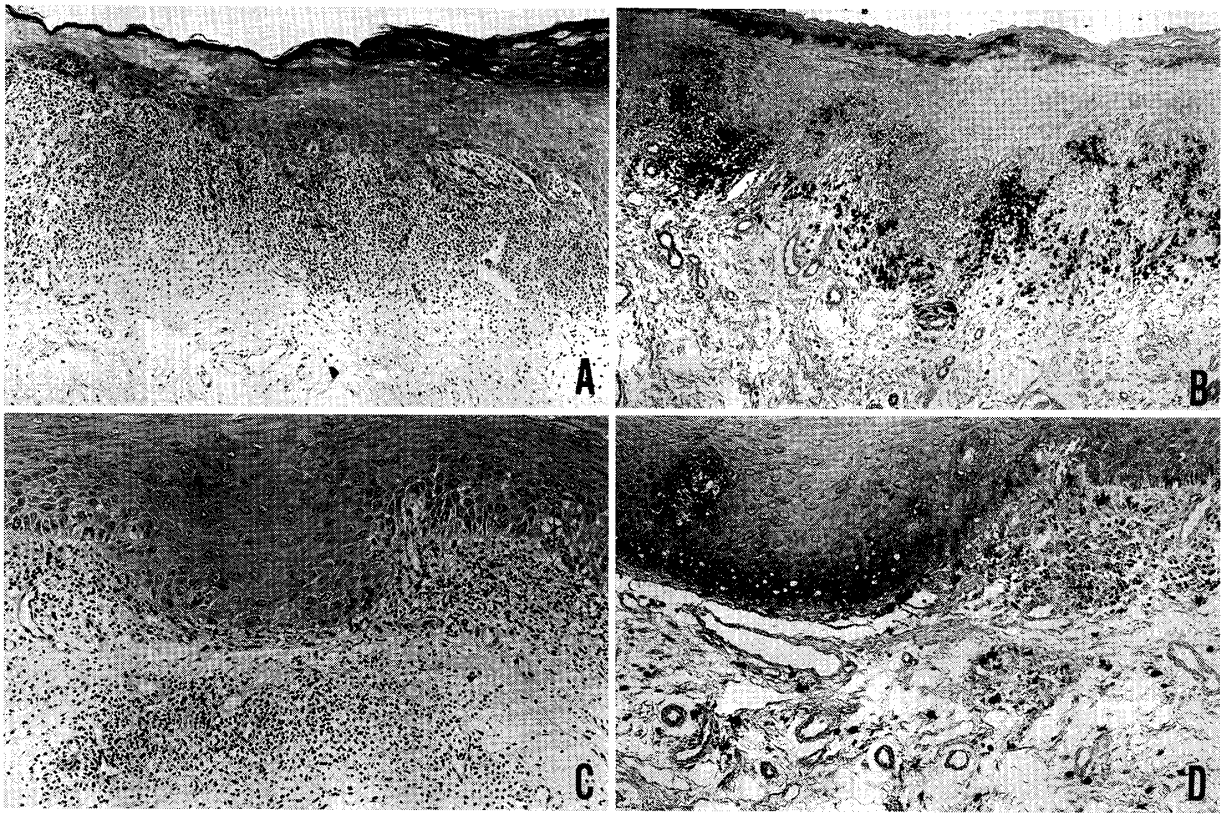

Fig. 3 Histopathology (A, C) and *in situ* hybridization (B, D) of oral lichen planus (OLP)
A, C: hematoxylin-eosin stain of oral lichen planus; original magnification ×100.
B: EBER-positive lymphocytes in lamina propria affected with OLP; original magnification ×100.
D: BHLF-positive lymphocytes in lamina propria affected with OLP; original magnification ×100.

DNA. EBER and BHLF hybridization signals were mainly localized in the infiltrating lymphocytes of the lesions. In two cases (OSCC and OL), EBER signals were also present in some basal cells of the oral mucosa epithelium. The proportion of EBER or BHLF-positive lymphocytes varied among the examined diseases, but OLP specimens expressed intense hybridization signals and a large propotion of EBER or BHLF-positive lymphocytes.

In the specimens of other diseases examined, the EBER or BHLF-positive cells were sparsely distributed throughout a small portion of the tissue. The labelling index was also significantly higher in OLP specimens (EBER, SD ± 36.8%) (BHLF, SD ± 16.4%) than in other diseases (EBER, SD ± 5～11%) (BHLF, SD ± 0～6%) (Table 2, Figs. 3～5).

Discussion

After primary infection with EBV, a life-long latent infection is established, reflecting a balance between viral replication and host immunity[13]. Because of the low copy numbers of EBV DNA, direct detection of EBV infected cells by nucleic acid hybridization techniques are difficult for latent infections[14]. Therefore, a more sensitive nested PCR technique was used, resulting in the detection of EBV DNA in 68.5% of the examined specimens. These results suggest that lymphocytes in the oropharyngeal region may serve as a virus reservoir for latent EBV infections. Similar findings describing a high detection rate (40～

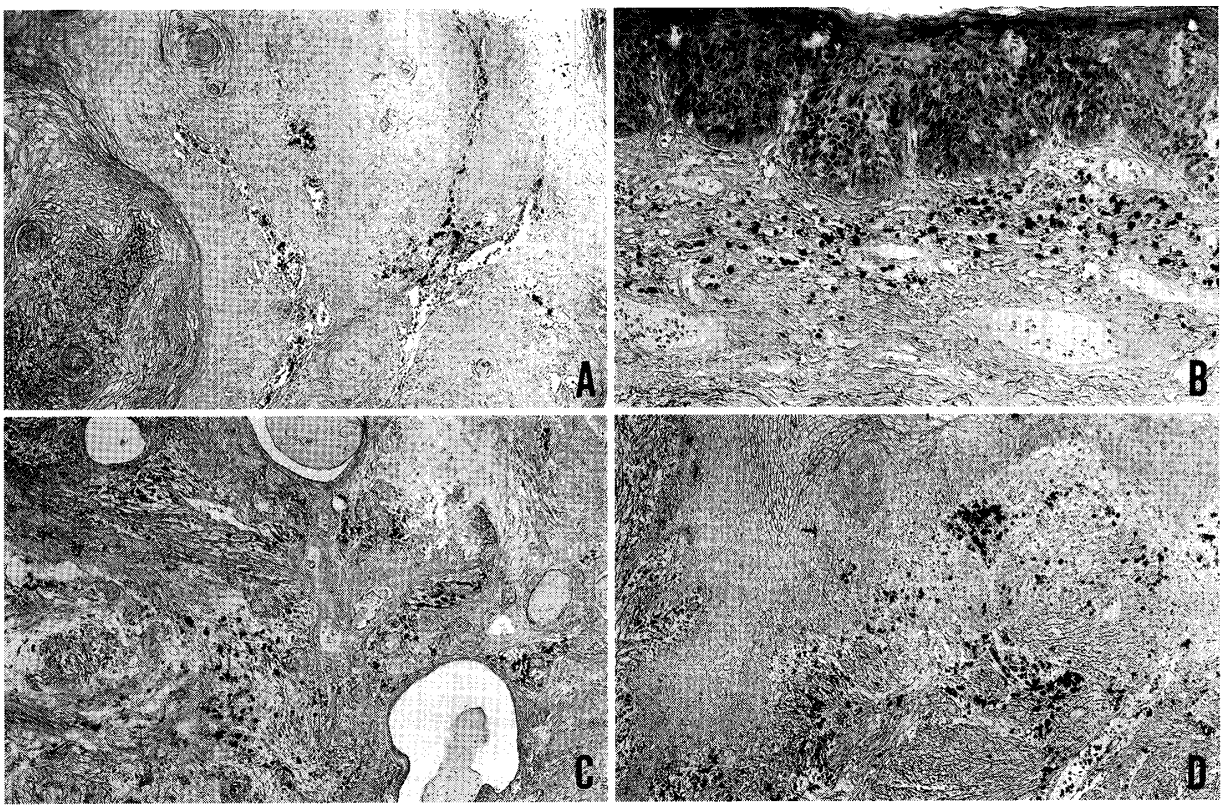

Fig. 4 EBER-positive lymphocytes in oral diseases, as examined by *in situ* hybridization
A: oral squamous cell carcinoma; original magnification ×100.
B: oral leukoplakia; original magnification ×100.
C: pleomorphic adenoma; original magnification ×100.
D: epulis; original magnification ×100.

86.7%) of EBV DNA in oral and pharyngeal tissues have been reported by other investigators[8)10)]. Our data disagree with the negative findings in some reports stating that EBV DNA is not detected in the oral mucosa[14)15)]. A direct comparison of their results and ours is difficult, however, because different regions of the virus were amplified and because the techniques that were used had different sensitivities.

To identify the cellular locations of the nested PCR signals, ISH was performed using PNA probes for EBER and BHLF mRNA. EBER are a good marker EBV infection because they are expressed at high levels in the nuclei of latent infected cells (10^6～10^7 copies per cell)[16)]. When an EBV latent infection becomes a productive lytic infection, the expression of EBER is down-regulated[17)18)] and EBER negative cells are not always uninfected cells as found in hairy leukoplakia in which the EBV replicating cells do not express EBER[19)]. For this reason, the presence of BHLF, which not only allows the detection of productive infected cells but of those cells that are in the process of switching from a latent to a productive infection, was also examined[15)]. Since PNA probes have several advantages, including a higher sensitivity and a shorter hybridization time, compared to oligonucleotide probes, the procedures could be completed within a day.

EBER mRNA were detected using ISH in many of the infiltrating lymphocytes, while BHLF was detected only in the lymphocytes. These observations disagree with the hypothesis that epithelial cells are susceptible to latent EBV

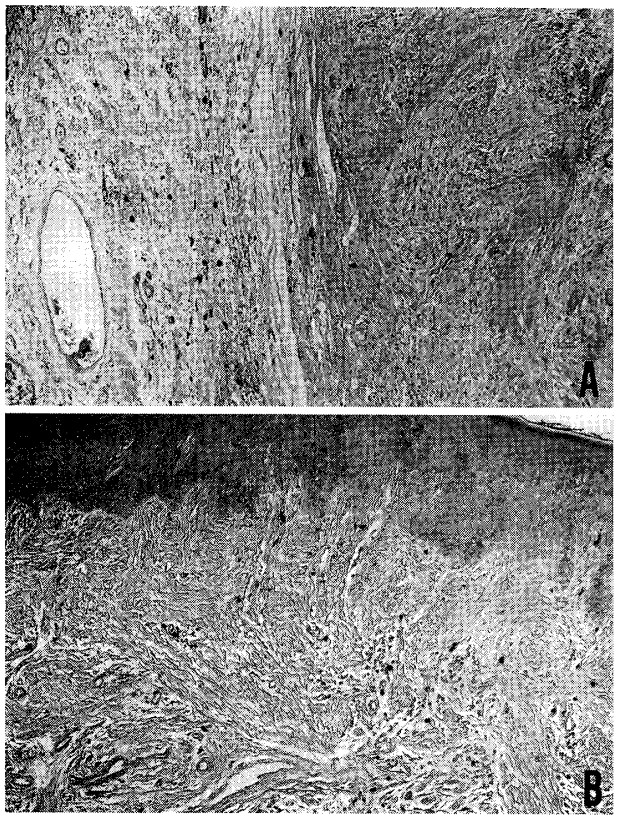

Fig. 5 BHLF-positive lymphocytes in the oral diseases, as examined by *in situ* hybridization
A: fibroma; original magnification ×100.
B: epulis; original magnification ×100.

infections, whereas B cells are secondarily infected with EBV[20)21)]. However, our results are consistent with a recent study showing that lymphocytes can harbour latent EBV infections and the productive lytic infections occur in lymphocytes but not in the oropharyngeal and nasopharyngeal epithelial cells[11)14)22)]. Spontaneous lytic replication in these lymphocytes may be the source of the virus that is detectable in saliva secretions.

Our findings that a larger number of lymphocytes express EBER and BHLF mRNA in OLP than in the other diseases that were examined, suggest that EBV-infected lymphocytes may be relevant to the pathogenesis of OLP. An aberration in the humoral response to EBV in OLP patients has been reported[23)] in a small controlled study that showed a higher prevalence of IgG antibodies to EBV early antigens in cases with OLP, but no significant correlations between the duration of the disease and the titer of IgG antibodies to EBV early antigens were found. A previous study using PCR and ISH to detect EBV DNA demonstrated positive signals in many of the lymphocytes and some of the epithelial cells in preulcerative oral lesions of patients with recurrent aphthoid ulcer or Behçet's disease, suggesting that the ulcers were formed when cytotoxic T lymphocytes attacked the EBV-infected cells and the mature epithelium[24)].

OLP, a chronic inflammatory disease with no well-defined etiology, is characterized by epithelial atrophy and hyperkeratosis, basal cell destruction, and a band-like dense infiltration of T lymphocytes[25)]. OLP is thought to be a T-lymphocyte-mediated autoimmune disorder, and epithelial damage in OLP is known to be directly caused by the infiltration of killer T-lymphocytes[26)27)]. From our histopathologic findings that a significantly high EBV infection rate is present in OLP specimens, we belive that the large number of EBV-infected cells in OLP lesions might induce the intense infiltration of T-cells and a high level of killer T-cell activity.

In conclution, we have demonstrated that oropharyngeal lymphocytes act as a reservoir for latent EBV infection and that EBV replication occurs in lymphocytes but not in epithelial cells. Furthermore, a significantly high level of EBER and BHLF expression suggests that EBV infections may be implicated in the pathogenesis of OLP.

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口腔疾患におけるEBウイルスの関与に関する検討

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EBVはヒトに極めて広範に常在するヘルペスウイルスであり，バーキットリンパ腫，上咽頭癌の病因ウイルスと考えられる．口腔領域においては免疫不全者の舌白板症，唾液腺腫瘍への関連が指摘されている．今回，我々は当科で治療を行った口腔腫瘍および粘膜疾患105例について，疾患とEBV感染の関連を検討するため，PCR法，PCR-Southern blot hybridization法，PNA probeを使用した*in situ* hybridization（ISH）法でEBVゲノムの検出およびmRNAの局在の検討を行った．

PCRでは口腔扁平上皮癌18/22例，線維腫11/21例，epulis 16/19例，白板症8/16例，多形性腺腫6/10例，乳頭腫6/9例，口腔扁平苔癬7/8例でEBVゲノムが検出され，EBV DNAの発現率は68.5%であった．ISHではEBVのEBERおよびBHLF mRNAの発現部位は主にリンパ球に観察された．EBERとBHLF陽性細胞の局在は調べた疾患によってまちまちであったが，口腔扁平苔癬にはEBERおよびBHLF陽性リンパ球のlabelling indexが他の疾患に比較して有意に高値を示した．

以上の結果より口腔組織中にEBVが高率に存在し，EBV感染細胞の局在は主にリンパ球であり，口腔扁平苔癬の病因にEBV感染が関与している可能性が示唆された．