

Involvement of Epstein-Barr virus (EBV)-encoded latent membrane protein (LMP)-1 in the potentiation of C ϵ mRNA expression in human tonsil-derived cells

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Abstract The aim of our study was to investigate a possible correlation between Epstein-Barr virus (EBV) infection status, including its latent gene expression, and expression of allergy-related genes in human tonsil-derived cells. In the tonsil-derived cells from the patients undergoing routine tonsillectomies for palatine tonsil hypertrophy or tonsillar focal infection, the presence of EBV DNA and mRNA expressions of latent membrane protein (LMP)-1, C ϵ chain, and activation-induced cytidine deaminase (AID) were detected by PCR and RT-PCR analysis, respectively. Of all the 12 patients, PCR products amplified from EBV DNA *Bam*HI W fragment were detected in the tonsils from the 10 patients (83.3%). LMP1 mRNA expressions were confirmed in the six patients (50%). Both LMP1 mRNA expressions and EBV DNA were detected in

the five patients. EBV DNA, but not LMP1 mRNA expression, was detected in the five patients. LMP1 mRNA expression, but not EBV DNA, was detected in one patient. In one patient, neither EBV DNA nor LMP1 mRNA expression was confirmed. C ϵ mRNA expressions were confirmed in all the 12 patients along with AID mRNA expressions. The degree of C ϵ mRNA expression, however, varied with the patients. The Fisher's exact probability test revealed a statistically significant correlation between LMP1 and C ϵ gene expressions, indicating that C ϵ mRNA expression level was significantly higher in the LMP1 positive samples than in the negative samples. On the other hand, there was no significant correlation between AID and LMP1 mRNA expressions. Thus, EBV infection is a notable factor capable of exacerbating allergic inflammation.

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Introduction

Although Epstein-Barr virus (EBV) infection appears to pass asymptotically when infected in childhood, exposure to EBV in adolescence occasionally causes infectious mononucleosis [3, 13]. EBV is also involved in several malignancies such as nasopharyngeal carcinoma, Burkitt's lymphoma, Hodgkin's disease, and gastric carcinomas [7]. EBV appears capable of infecting two major cell types such as epithelial cells of the nasopharynx and B cells. EBV, which establishes a long-lasting latent infection within B cells in the vast majority of human population, is reportedly involved

in exacerbation of allergic reactions. Activation of antigen-presenting cells by EBV infection is known to lead to promoting allergic inflammation [10, 13]. Increased levels of EBV antibodies have reportedly been found both in child patients with asthma and atopic dermatitis [14, 15]. In vitro experiments indicate that EBV is a stimulator of the production of IgE, which is a key molecule in the initiation of type I allergic reaction. EBV-transformed B cells stimulated with interleukin-4 (IL-4) produce IgE in culture supernatants [9, 16], and the level of IgE secreted in culture supernatants correlates with the number of IgE-producing cells [9].

Epstein-Barr virus is a common herpes virus present in almost all human populations. The latent gene products encoded by EBV are classified into four groups so far: (1) six nuclear antigens (EBNA1, EBNA2, EBNA3A, EBNA3B, EBNA3C, and EBNA leader protein); (2) three latent membrane proteins (LMP1, LMP2A, and LMP2B); (3) small non-polyadenylated RNAs (EBERs 1 and 2); and (4) EBV *Bam*HI-A rightward transcripts (BARTs) [21]. Of these latent proteins (except EBERs and BARTs), the LMP1, which is a receptor-like protein constitutively activated, leads to elicitation of several biological effects including B-cell transformation.

Latent membrane protein-1, a 63-kDa integral membrane protein consisting of three domains, has within its C-terminus two activating regions referred to as C-terminal activating region-1 (CTAR1) and CTAR2 [3]. LMP1 can also protect the B cell from apoptosis by inducing anti-apoptotic proteins including BCL-2, MCL-1, and A20 [3]. LMP1 is known to mimic CD40 through activating the overlapping signaling pathways between them [4, 17]. The key molecules for the signaling by both LMP1 and CD40 are considered to be the intracellular tumor necrosis factor (TNF) receptor-associated factor (TRAF) adapter proteins, which are recruited to the cytoplasmic C-terminus domains of both LMP1 and CD40 [4]. LMP1 signaling is, however, considered to be distinct from that of CD40, because it is a receptor-like molecule, which is constitutively activated without ligand binding [8].

CD40, which belongs to the TNF-receptor family, is a membrane protein found on the surface of B cells and serves as the receptor for CD40 ligand expressing on the cell surface of T cells. CD40 mediates activation of several transcription factors including nuclear factor- κ B (NF- κ B) and signal transducers and activators of transcription (STAT) molecules, thereby leading to B-cell proliferation, antibody class switching, and modulation of apoptosis. Activation of CD40 signaling pathway in the presence of IL-4 or IL-13 leads to expression of activation-induced cytidine deaminase

(AID) [20]. This novel RNA-editing enzyme plays a key role upstream of the putative switch recombinase, leading to IgE isotype switching, mature C ϵ transcription, and IgE synthesis [12, 20].

We herein focused on the tonsil-derived cells, because (1) the tonsils are readily available for study and appear to be one of the first targets of infection by EBV, which is also known as a causative agent involved in the development of recurrent tonsillitis and tonsillar hypertrophy [19]; and (2) the tonsils function as a important tissue for the immune system, where immunocompetent cells including B cells grow and differentiate. The aim of the present study was to investigate a possible correlation between EBV infection status including its latent gene expression and expression of IgE and AID in human tonsil-derived cells. The expression of IgE mRNA was measured as the expression of C ϵ (constant region of ϵ -heavy chain of IgE) mRNA.

Materials and methods

Subjects

Palatine tonsils were obtained from 7- to 47-year-old patients (eight males and four females) undergoing routine tonsillectomies for palatine tonsil hypertrophy with obstructive sleep apnea syndrome or for tonsillar focal infection at the University of the Ryukyus Hospital (Table 1).

Preparation of tonsil-derived cells

Tonsillar tissues were employed for the study after obtaining informed consent from the patient or the patients' parents. We followed the ethical principles for medical research involving human subjects (World Medical Association Declaration of Helsinki) for proceeding to the present research, together with the requirements of the ethical review committee in the University of the Ryukyus. Routine histological analysis of tonsillar tissues indicated no involvement of malignancies. Tonsils were minced with the scissors in ACT buffer (containing 25 mg/ml of D-glucose and 19 mg/ml of sodium citrate), then placed and mashed between two slide glasses to remove highly adherent cells such as macrophages. The resulting suspension was passed through a stainless steel wire mesh to remove any connective tissue. After the cell suspension was centrifuged at 1,000 rpm for 10 min at 4°C, the cell pellet was suspended in 0.17 M NH₄Cl solution for 15 min at room temperature to destroy erythrocyte

Table 1 List of patients studied

	Case no.	Age	Gender	Clinical diagnosis
Palatine tonsils were obtained from 7- to 47-year-old patients undergoing routine tonsillectomies for palatine tonsil hypertrophy with obstructive sleep apnea syndrome or for tonsillar focal infection at the University of the Ryukyus Hospital	1	44	M	Tonsillar focal infection with pustulosis palmoplantaris
	2	35	M	Tonsillar focal infection with IgA nephropathy
	3	22	M	Tonsillar focal infection with IgA nephropathy
	4	7	M	Tonsil hypertrophy with obstructive sleep apnea syndrome
	5	31	M	Tonsillar focal infection with IgA nephropathy
	6	23	M	Tonsillar focal infection with IgA nephropathy
	7	24	M	Tonsillar focal infection with Henoch-Schonlein purpura
	8	19	F	Tonsillar focal infection with IgA nephropathy
	9	43	F	Tonsillar focal infection with IgA nephropathy
	10	47	M	Tonsillar focal infection with pustulosis palmoplantaris
	11	36	F	Tonsillar focal infection with IgA nephropathy
	12	36	F	Tonsillar focal infection with erythema nodosum

M male, *F* female

membranes, followed by centrifugation. The cell pellet was then washed in RPMI 1640 medium (Nissui Pharmaceutical Co., Tokyo, Japan) without serum, followed by centrifugation, and employed for the following experiments. Cells derived from EBV-negative tonsils were used as negative controls for all reverse transcriptase-polymerase chain reaction (RT-PCR) experiments.

DNA and RNA isolation

Genomic DNAs and total RNAs were prepared from tonsil-derived cells using the PUREGENE[®] DNA Purification Kit and PURESRIPT[®] RNA Isolation Kit (Gentra Systems, MN, USA), respectively, according to manufacturer's instructions.

Reverse transcription of mRNA and polymerase chain reaction

Reverse transcriptions were carried out according to the standard procedures. In brief, 1 µg of total RNA in 18.8 µl of reaction mixture (4 µl of 5 × RT buffer, 4 µl of 2.5 mM dNTP mixture, 2 µl of 25 mM MgCl₂, 2 µl of 0.1 M DTT, and RNase free water as the rest of mixture) and 0.2 µl of 50 µM synthetic oligo(dT) primer were mixed. After denaturing at 75°C for 3 min, the reaction was cooled down on ice and initiated by adding 1 µl of M-MLV RTase and incubated for 1 h at 42°C.

The cDNAs of LMP-1, Cε, AID, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) genes were amplified by the PCR method with Program Temp Control System PC-800 (Astec Inc., Fukuoka, Japan). The reaction was performed in a final volume of 20 µl of reaction mixture, which consisted of 20 mM Tris-HCl pH 8.4, 50 mM KCl, 1.5 mM MgCl₂, 0.2 mM dNTPs, 0.5 µM each primer, 1 µl of *Taq* DNA polymerase (Promega, WI, USA) (final concentrations), and 1 µl of sample. Primers for PCR were as follows: LMP1 [1], forward, 5'-TTTGTCTACTCCTACTGATGATCACC-3', and reverse, 5'-AGTAGATCCAGAGACCTAAGACAAGT-3'; Cε: forward, 5'-TACCTGGTCTGCTGGTCTCC-3', and reverse, 5'-GAGGACTGGTGTGGGTCAAG-3'; AID, forward, 5'-GGCTGCCACGTGGAATTG-3', and reverse, 5'-CTTCC CAGGCTTTGAAAG-3'; GAPDH, forward, 5'-GT CAGTGGTGGACCTGACCT-3', and reverse, 5'-G ACTGAGTGTGGCAGGGACT-3'. The reaction mixtures were incubated at 94°C for 2 min. The conditions for PCR besides annealing condition were as follows: 94°C for 1 min for denaturation; 74°C for 1 min for extension; 30 cycles (40 cycles for LMP1; 26 cycles for GAPDH) for repetition of reaction. Annealing conditions for PCR were as follows: for LMP1, 58°C for 1 min; for Cε, AID, and GAPDH, 60°C for 1 min. All PCRs were concluded with a 5-min incubation at 74°C to complete the extension of all synthesized products. PCR products were run on a 2%-agarose gel, visualized with ethidium bromide, and analyzed using

the Gel Doc 1000 UV fluorescent gel documentation system (Bio-Rad Lab., CA, USA) with the accompanying Multi-Analyst software (version 1.0).

PCR without reverse transcriptase (DNA-PCR)

DNA-PCR was carried out for detecting EBV DNA. Twenty nanogram of sample DNA was employed for PCR under the same amplification conditions as used for RT-PCR unless specifically mentioned. For amplification of the *Bam*HI W sequence of EBV DNA, we used the following primers [18]: forward, 5'-CCAGAC AGCAGCCAATTGTC-3', and reverse, 5'-GGTAG AAGACCCCCTCTTAC-3'. The conditions for PCR were: 94°C for 1 min for denaturation; 55°C for 1 min for annealing; 74°C for 1 min for extension; 40 cycles for repetition of reaction.

Statistical analysis

Fisher exact probability test was used to analyze a 2×2 contingency table of categorical data. A *P* value of <0.05 was taken as significant.

Results

Detection of EBV DNA and allergy-related gene expressions in cells derived from tonsils

Epstein-Barr virus DNA level and mRNA expression levels of Cε, AID and LMP1 in the tonsil-derived cells were categorized according to the intensity of PCR products analyzed on an agarose-gel (Table 2). Of the 12 cases, PCR products amplified from EBV DNA *Bam*HI W fragment were detected in the tonsils from ten patients (83.3%). LMP1 mRNA expressions were confirmed in the 6 patients (50%) out of 12 patients. Both LMP1 mRNA expression and EBV DNA were detected in five patients. EBV DNA, but not LMP1 mRNA expression, was detected in five patients. LMP1 mRNA expression, but not EBV DNA, was detected in one patient. In one patient, neither EBV DNA nor LMP1 mRNA expression was confirmed (Fig. 1 and Table 2).

Expression of allergy-related genes

We focused on Cε and AID genes as allergy-related genes, and their expression was determined by PCR analysis. Cε mRNA expressions were confirmed in the all the 12 patients as well as AID mRNA expressions. The degree of Cε mRNA expression, however, varied among the 12 patients (Fig. 1 and Table 2).

Table 2 Summary of Epstein-Barr virus (EBV) infection status and expression pattern of allergy-related genes in tonsil-derived cells

Case no.	EBV DNA	LMP1	Cε	AID
1	1	0	1	1
2	1	0	1	1
3	0	1	2	1
4	1	2	2	1
5	0	0	1	1
6	2	0	1	2
7	1	2	2	1
8	2	2	2	1
9	2	0	2	1
10	1	0	1	1
11	1	1	2	1
12	1	1	2	1

EBV DNA level and mRNA expression levels of Cε, AID, and LMP1 in the tonsil-derived cells were classified into three classes according to the intensity, which is represented by arbitrary units, of PCR products analyzed on an agarose-gel electrophoresis. Class 0: <500, class 1: 500–30,000, class 2: >30,000

Correlation between LMP1 and Cε mRNA expressions in cells derived from tonsils

The null hypothesis was herein that the two populations of LMP1 positive and negative samples were homogeneous at Cε mRNA expression levels. We therefore rejected the null hypothesis on the basis of Fisher's exact test result, *P* = 0.0152, and concluded that the two populations were different (Table 3). The result suggests the possibility that LMP1 expression is involved in Cε mRNA expression in the tonsil-derived cells. On the other hand, there is no significant relation between AID and LMP1 mRNA expressions (data not shown).

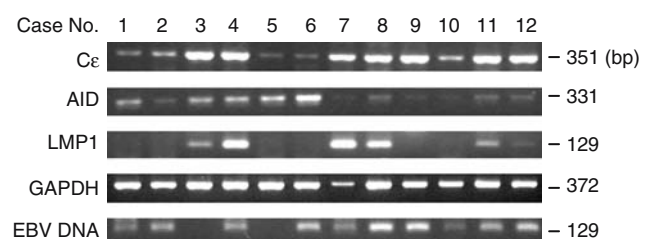


Fig. 1 Detection of Epstein-Barr virus (EBV) DNA and allergy-related gene expressions in tonsil-derived cells. RT-PCR analysis for determining mRNA expressions of Cε chain, activation-induced cytidine deaminase (AID), and EBV latent gene LMP1 was performed on cells derived from tonsils. EBV DNA was amplified with PCR using a set of primer for *Bam*HI W fragment. The migration points for the PCR products indicated by arrows and their product sizes are shown

Table 3 Correlation between LMP1 and Cε gene expressions in tonsil-derived cells

	Cε		Total
	Class 1	Class 2	
LMP1			
Negative	5	1	6
Positive	0	6	6
Total	5	7	12

Data in the table represent the number of patients. The null hypothesis is that the two populations of LMP1 positive (class 1 or class 2) and negative (class 0) samples are homogeneous at Cε gene expressions. Fisher's exact test result, $P = 0.0152$

Discussion

We demonstrated that EBV DNA were detected in 83.3% and LMP-1 mRNA expressions were detected in 50% of the patients tested. Cε mRNA expressions were confirmed in all the 12 patients along with AID mRNA expression. We further examined the correlation between LMP1 and Cε gene expression levels in the tonsil-derived cells. The results revealed that Cε gene expression levels were higher in the patients with LMP1 positive than in the patients with LMP1 negative, indicating that IgE gene expression is associated with LMP1 expression.

Previous studies to clarify the correlation between EBV infection and serum IgE levels, however, yielded controversial and conflicting results. Rystedt et al. [14] indicated that there was no correlation between EBV antibody titers and serum IgE levels. Calvani et al. [6] reported that the prevalence of high IgE levels was more frequent in the EBV negative than in the positive subjects. They also indicated that this higher prevalence in the EBV negative subjects was found only in the groups aged under six, whereas in the 6–19 year group the situation was reversed [6]. In contrast, in the heterophil-positive infectious mononucleosis, high serum IgE level was reportedly observed very early in the course of the disease, mainly within first week [2]. A strong correlation between the IgE response and the number of circulating atypical lymphocytes was also confirmed in the heterophil-positive infectious mononucleosis [2]. Based on these reports, it is anticipated that the host's immunoresponsiveness to virus infection, which varies with the age of patients and with the passage of time since the primary infection, seems to influence the correlation between EBV infection and the serum IgE level.

The immune system involved in Cε gene expression, including germline transcription and gene rearrangement, might be promoted by LMP1 gene expression,

thereby leading B cells to differentiation toward IgE-producing plasmacytes. LMP1 is known to accomplish its function by signaling through several different pathways: the NF-κB pathway, the c-Jun N terminal kinase [(JNK)-AP-1] pathway, the p38/mitogen activated protein kinase (MAPK) pathway, and the Janus kinase [(JAK)-STAT] pathway [3, 11]. Therefore, LMP1 is predicted to be involved in IgE gene expression by activating NF-κB pathway and/or JAK-STAT pathway. On the other hand, AID plays a key role in immunoglobulin class switch, leading us to the hypothesis that Cε gene expression is promoted by LMP1 gene expression through AID gene expression. However, no significant relation was observed between AID and LMP1 gene expressions. Therefore, it is unlikely that Cε gene expression induced by LMP1 is due to LMP1-induced AID gene expression. In the present study, LMP1 gene expression was not observed in five out of ten patients, who have EBV DNA in the tonsil-derived cells. The pattern of latent gene expression is known to vary by its target cells and tissues [5, 21]. Whether or when LMP1 gene expresses following the first EBV infection might depend on the host's immunoresponsiveness to the virus infection, which is possibly associated with patient age and/or the expression status of other latent genes.

Epstein-Barr virus infects the oropharynx tissues such as tonsils and salivary glands as its primary target tissues. Although EBV is ordinarily dormant in the host forever after primary infection, it sometimes allows the infected cells to acquire the unlimited growth potential, thereby resulting in several malignancies. We have found in the present study a positive correlation between EBV-LMP1 and Cε mRNA expressions in human tonsil-derived cells, suggesting that EVB infection is a notable factor capable of exacerbating allergic inflammation. One possible mechanism underlying the exacerbation of allergic inflammation by EBV is that LMP1 gene expression activates B cells and enables them to differentiate into IgE-producing plasmacytes, thereby enhancing IgE levels and potentiating allergic reactions.

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