Research Communication

Modification of $\text{C} \varepsilon$ mRNA Expression by EBV-Encoded Latent Membrane Protein 1

Kazuhiko Hanashiro,¹ Shigeto Ohta,² Masanori Sunagawa,¹ Mariko Nakamura,¹ Mikio Suzuki,² and Tadayoshi Kosugi¹

¹ 1st Department of Physiology, School of Medicine, University of the Ryukyus, 207 Uehara, Nishihara, Okinawa 903-0215, Japan
² Department of Otorhinolaryngology-Head and Neck Surgery, School of Medicine, University of the Ryukyus, 207 Uehara, Nishihara, Okinawa 903-0215, Japan

INTRODUCTION

Epstein-Barr virus (EBV) is a member of the human herpesvirus family and latently infects more than 90% of the world’s adult population. EBV infects the oropharynx tissues such as tonsils and salivary glands as its primary target tissues. Although EBV is ordinarily dormant in the host for over 90 years after primary infection, it sometimes allows B cells to acquire the unlimited growth potential. Therefore, lymphoma would be observed in the case of the weak host’s immunoresponsiveness to the virus infection. EBV is also reportedly involved in allergic reactions. Increased levels of EBV antibodies are found both in children with asthma and atopic dermatitis [1, 2]. In in vitro studies, EBV-transformed B cells stimulated with IL-4 produce IgE in culture supernatants [3, 4], and the level of IgE secreted in culture supernatants correlates with the number of IgE-producing cells [4]. Transformation of B cells by EBV has been reported to increase the number of IgE-producing cells [5]. Clinical studies to clarify the correlation between EBV infection and serum levels of IgE, which play a pivotal role in allergic reaction, yielded however controversial and conflicting results [1, 2, 6, 7]. The mechanisms underlying the causal relationship between the EBV infections and the increase of the risk for allergic diseases remain to be fully elucidated. There are however two possible mechanisms underlying there. One of them is through activating antigen-presenting cells by secretion of several cytokines including tumor necrosis factor-α (TNFα), interferon-γ (IFN-γ), and interleukin-1α (IL-1α) from EBV-activated T cells [8]. Another one is through triggering T-cell independent class switch DNA recombination (CSR) by EBV-latent membrane protein-1 (LMP1) [9]. LMP1-induced CSR in B cells has been reported to be associated with transcriptional activation of germline $\text{C} \gamma$, $\text{C} \alpha$, and $\text{C} \varepsilon$ genes and to trigger the upregulation of activation-induced cytidine deaminase (AICD), a crucial component of the CSR machinery [9]. However, it is yet uncertain how the differentiation status of B cells, where EBV-LMP1 is expressed, is associated with their ability to produce IgE.

The latent gene products encoded by EBV are classified into four groups, and at least 9 proteins have been identified so far [10]. Of these latent proteins, the LMP1 is a cell surface protein, which is a receptor-like protein constitutively activated, leading to elicitation of several biological effects including B-cell transformation. LMP1, 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 [11]. These CTARs are involved in activation of four different signaling pathways of LMP1, including the nuclear factor
κB (NF-κB) pathway [11], which potentially activates immunoglobulin gene expression [12]. LMP1 can also protect the B cell from apoptosis by inducing antiapoptotic proteins such as BCL-2, MCL-1, and A20 [11]. Therefore, LMP1 can be regarded as the EBV-encoded protein with the highest potentiality to modulate the immune system involved in IgE synthesis.

Brown-Norway (BN) rat is a useful experimental animal for studies on transplantation immunity, heredity, reproduction, aging, and immunoallergy. The BN rat is especially available for a model of allergic inflammation such as allergic rhinitis, asthma, and food allergy, because it is a high-socially available for a model of allergic inflammation such as allergic rhinitis, asthma, and food allergy, because it is a high-synthetic.

In the present study, we examined whether or not LMP1 modulates IgE production in rabbit antibodies against IgE FE-3 overnight at 4°C [13, 14]. In brief, the ELISA plate was coated with 5 μg/mL of rabbit antibodies against IgE FE-3 overnight at 4°C. After blocking with 1% gelatin-PBS, the wells were incubated with the rat serum appropriately diluted with 1% gelatin-PBS containing 0.05% Tween 20 (PBST) for 1 hour at room temperature. Following washing, the wells were incubated with 1 μg/mL of biotinylated DNP-As in 1% gelatin-PBST for 30 minutes at room temperature. After further washing, the wells were incubated with POD-conjugated streptavidin (ZYMED Laboratories, Calif, USA) in 1% gelatin-PBST for 30 minutes at room temperature, followed by final washing and addition of substrate solution (ABTS) (Sigma-Aldrich, Mo, USA). Spectrophotometric readings were then made using λ1 = 415 nm and λ2 = 492 nm wavelength filters of a dual wavelength microplate reader MTP-300 (CORONA Electric, Ibaraki, Japan).

**Preparation of splenocytes**

Nonimmunized and immunized BN rats were anesthetized with sodium pentobarbiturate, and the spleen was excised immediately after the rats had been killed by means of bleeding from the carotid artery. The immunized rats were employed for the experiments 7 days after the first immunization. The excised spleen was further torn to pieces, and then splenocytes were collected by centrifugation after removing these pieces.

**Plasmid and transfection**

pSG5-LMP1 was a gift of Dr Kieff (Harvard Medical School, Mass, USA). The vector was transfected into FE-3 cells or splenocytes derived from BN rats using DMRIE-C reagent (Invitrogen, Calif, USA) as follows. 0.5 mL of RPMI 1640 medium (Nissui Pharmaceutical Co, Tokyo, Japan) without serum (SFM) was added to each well of a 6-well plate. Then, 0.8 μL of DMRIE-C reagent was added to each well, and mixed gently by swirling the plate. Furthermore, 0.5 mL SFM containing 0.4 μg DNA was added to each well and then mixed by swirling the plate. The plate was incubated at room temperature for 30 minutes to allow formation of the lipid-DNA complexes. 2 × 10⁶ cells of FE-3 cells or 2 × 10⁷ cells of rat splenocytes were placed in the each well in which the plate was incubated at room temperature. Following washing, the wells were incubated with POD-conjugated streptavidin (ZYMED Laboratories, Calif, USA) in 1% gelatin-PBST solution containing 0.05% Tween 20 (PBST) for 1 hour at room temperature. Following washing, the wells were incubated with POD-conjugated streptavidin (ZYMED Laboratories, Calif, USA) in 1% gelatin-PBST for 30 minutes at room temperature. After further washing, the wells were incubated with POD-conjugated streptavidin (ZYMED Laboratories, Calif, USA) in 1% gelatin-PBST for 30 minutes at room temperature, followed by final washing and addition of substrate solution (ABTS) (Sigma-Aldrich, Mo, USA). Spectrophotometric readings were then made using λ1 = 415 nm and λ2 = 492 nm wavelength filters of a dual wavelength microplate reader MTP-300 (CORONA Electric, Ibaraki, Japan).

**RNA isolation and Northern blot analysis**

Total RNA (10 μg), prepared using PURESCRIPT RNA Isolation Kit (Gentra System, Minn, USA), was resolved by electrophoresis under denaturing conditions and transferred to a
nylon membrane. Ribosomal RNA was stained on the membrane with methylene blue to assess RNA loading and transfer efficiency. The procedure of hybridization was followed by instruction manual directed by Clontech. The Ce and β-actin cDNAs for probe were prepared by RT-PCR. The LMP1 cDNA for probe was obtained as a restriction fragment of the pSG5-LMP1. The probes for Northern blot analysis were then labeled with (α-32P)dCTP using random oligo-primed reaction.

Reverse transcription of mRNA

Reverse transcriptions (RTs) 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 5x 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 minutes, the reaction was cooled down on ice and initiated by adding 1 µL of M-MLV RTase and incubated for 1 hour at 42°C.

Polymerase chain reaction (PCR)

The cDNAs for Ce, LMP1, and β-actin 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 of each primer, 1 µL of Taq DNA polymerase (Promega, WI, USA) (final concentrations), and 1 µL of sample. Primer sequences are as follows: Ce [16], forward, 5’ CTTACCTGTCTGGTTTGGACCTGG 3’, and reverse, 5’ CGGAGGGAAGTTCAACCGGC 3’; β-actin [16], forward, 5’ GAGCTATGAGCTGCCTGACGG 3’, and reverse, 5’TTCGGTGACGATGGA 3’; and LMP1 [17], forward, 5’ TTGTTCACTCTACTGATGATCACC-3’, and reverse, 5’ AGTAGATCCAGACCTAAGACAAGT-3’. The PCR conditions were as follows: a single 5-minute at 94°C; 35 cycles (30 cycles for LMP1; 25 cycles for β-actin) of 94°C for 1 minute, 58°C for 1 minute, and 72°C for 1 minute; and a single 5-minute extension at 72°C. 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, Calif, USA) with the accompanying Multi-Analyst software (version 1.0).

Statistical analysis

Statistical analysis was performed by unpaired Student t test for between-group comparisons. Data are expressed as the means ± standard deviations (SD). When P < .05 was obtained, the means were considered to be significantly different.

RESULTS

Preparation of splenocytes from BN rats following immunization with DNP-As

Before collecting splenocytes from the immunized rats, we firstly determined to when the splenocytes have to be collected following immunization, based on the changes of IgE levels determined by ELISA. IgE levels in the immunized rats began to rise at day 6, and reached a peak at day 8 as shown in Figure 1. The immunocompetent cells with diversity in their maturity would exist on the initial stage after booster injection. This stage is also predicted to be essential for B cells to be destined to become the IgE-producing plasmacytes. The splenocytes were therefore collected at the early stage, day 7, where IgE production was observed to start increasing in the rat.

Effect of LMP1 expression on Ce mRNA levels of splenocytes derived from BN rats without immunization with DNP-As

The splenocytes derived from nonimmunized rats were transfected with the expression vector pSG5-LMP1, and then cultured in the presence or absence of rat IL-4. Because Ce mRNA in the splenocytes derived from nonimmunized BN rats was not detected on Northern blot analysis, the effect of LMP1 expression on the Ce mRNA levels in the cells was examined by RT-PCR analysis. Since the primers for RT-PCR amplification of Ce mRNA can amplify both germline and mature Ce transcripts, the PCR bands represent the total amount of germline and mature Ce transcripts. The splenocytes even without treatment with IL-4 showed as high levels of Ce mRNA as the cells treated with IL-4, suggesting that the function of B cells was regulated by IL-4 derived from T-cell population in the splenocytes collected. Ce mRNA levels were higher in the cells transfected with pSG5-LMP1 than those in the control cells transfected with the empty vector pSG5 (P < .05, Figure 2).

Effect of LMP1 expression on Ce mRNA levels of splenocytes derived from BN rats immunized with DNP-As

We next determined Ce mRNA levels in the splenocytes collected form the BN rats immunized with DNP-As on Northern blot analysis. Theoretically, (α-32P)-labeled probe for Ce transcripts can hybridize with both germline and mature Ce transcripts. The Ce mRNA band could be detected as a single band, which presumably represents mature Ce transcript based on its size of length. The results indicated that Ce mRNA levels were lower in the cells transfected with pSG5-LMP1 than those in the control cells transfected with pSG5 (Figure 3).
Figure 1: Changes of IgE levels in BN rats following immunization with DNP-As. The BN rats were injected with 1 mg of DNP-As together with killed Bordetella pertussis (10¹⁰ cells), followed by a single booster injection of DNP-As (0.5 mg) 5 days after the first injection. Blood samples were taken from the tail veins of the rats at 6, 7, 8, and 10 days after the first injection. Serum IgE levels in the rats were determined by IgE-capture ELISA as described in the “materials and methods.” The results were expressed as means ± SD of triplicate experiments.

Effect of LMP1 expression Cε mRNA levels of IgE-producing hybridoma, FE-3 cells

FE-3 cells are an IgE-producing hybridoma established by hybridizing mouse myeloma cells (SP2/0-Ag14/SF) with rat splenocytes that were isolated from BN rats immunized with DNP-As [13]. The cDNA sequence of the Cε3 and Cε4 domains of IgE FE-3 is identical to that of rat IgE IR162 [15], indicating that IgE obtained from the hybridoma cells is encoded in rat immunoglobulin locus. Our Southern blot analysis for the genomic DNA of the FE-3 cells indicates that switch recombination in the Cε gene locus is accomplished in the cells (our unpublished data). FE-3 cells are therefore expected to be available for various allergic experiments as a model of plasmacytes that constitutively produce IgE antibodies. As shown in Figure 4(a), two bands are seen in Northern blot. The faster migrating band corresponds to the mRNA for the secreted form of IgE, whereas the slower band for the membrane-bound form of IgE. Following the transfection of FE-3 cells with pSG5-LMP1 or pSG5 empty vector, there is no significant difference in IgE mRNA levels between the cells transfected with pSG5-LMP1 and the control cells transfected with pSG5 (Figure 4).

DISCUSSION

In the present study, we found that the effect of LMP1 overexpression on the Cε gene expression in the splenocytes was different depending on the immunization condition of BN rats. Although IL-4 failed to augment Cε mRNA expression, overexpression of LMP1 upregulated Cε mRNA expression in splenocytes derived from nonimmunized rats (Figure 2). This indicates that LMP1 upregulates Cε mRNA expression independently of stimulation by IL-4. Overexpression of LMP1 and Cε mRNA expression were determined by Northern blot analysis as described in the “materials and methods.” The results of three independent experiments are shown.
LMP1, however, abrogated Cε mRNA expression in splenocytes derived from immunized rats (Figure 3). The underlying mechanism for negative effects of LMP1 on the B cells differentiating toward IgE-producing cells is yet unidentified. The positive effects of LMP1 expression on the B cells that have no definitive direction of differentiation, however, might be due to the promotion of germline Cε transcription and Cε gene rearrangement by LMP1 expression. In IgE-producing hybridoma FE-3 cells, which had accomplished gene rearrangement in Cε locus, Cε mRNA levels were not altered by overexpression of LMP1 (Figure 4).

Increased levels of EBV antibodies are found both in children with asthma and atopic dermatitis [1, 2], suggesting EBV infection might promote allergic disease. The correlation between EBV infection and serum IgE level is, however, controversial and still need to be clarified. Rystedt et al indicated that there was no correlation between EBV antibody titers and serum IgE levels [2]. Calvani et al reported that the prevalence of high IgE levels was more frequent in the EBV negative than in the positive subjects [7]. They also indicated that this higher prevalence in the EBV negative subjects was found only in the groups of five years old or less, whereas in the 6–19 years group the situation was reversed [7]. On the contrary, heterophil-positive infectious mononucleosis is found to be associated with high serum IgE level [6]. In addition, the increase in IgE level was observed very early in the course of the disease, mainly within the first week, rapidly followed by a significant drop below the preillness levels [6]. Based on these studies, it is anticipated that the host’s immunoresponsiveness to virus infection, which varies in patient age and the passage of time from the primary infection, seems to influence the correlation between EBV infection and the serum IgE level.

The B-cell activating signal for IgE synthesis is derived through CD40 ligand and IL-4 or IL-13. Both IL-4 and IL-13 are capable of inducing germline Cε transcription in B cells, because IL-4 and IL-13 receptors share the IL-4Ra chain, which plays a pivotal role in the induction of germline Cε transcription, as the common component of both receptors [18, 19]. Target sites for several transcription factors have been identified in the lε promoter, including STAT6, NF-κB, PU.1, BSAP, C/EBP (for human) [20–22], and AP-1 (for mouse) [23, 24]. IL-4 and IL-13 are known to induce activation of STAT6 [20, 22], which promotes RNA transcription at the Cε locus and is therefore regarded as the critical regulator for germline Cε transcription. 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 the activation of several transcription factors including NF-κB and STAT molecules [25], 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 the expression of AICD [26]. This novel RNA-editing enzyme plays a key role upstream of the putative switch recombinase, leading to IgE isotype switching, mature Cε transcription, and IgE synthesis [12, 27]. Thus, IL-4Ra, which is involved in the activation of overlapping signaling pathways between IL-4 and IL-13, and CD40 signaling pathways are integrated to induce IgE isotype switching, followed by IgE synthesis. ε germline gene transcription, class switch recombination, and splicing of ε-chain gene transcript in the course of IgE synthesis are considered as phenomena synchronized with the differentiation process of B cell.

A strong correlation is noted between the IgE response and the number of circulating atypical lymphocytes in the heterophil-positive infectious mononucleosis [6]. In vitro experiments indicate that EBV is an enhancer of IgE production. EBV-transformed B cells stimulated with IL-4 are allowed to produce IgE in culture supernatants [3, 4], and the level of IgE secreted in culture supernatants correlates with the number of IgE-producing cells [4]. LMP1 is most likely the EBV-encoded protein with highest activity to modulate IgE production, because LMP1 is known to mimic CD40 through activating the overlapping signaling pathways between them. He et al reported that LMP1 triggered T-cell independent CSR [9]. LMP1-induced CSR is associated with transcriptional activation of germline Cγ, Cα, and Cε genes and triggers the upregulation of AICD [9].
The key molecules for the signaling by both LMP1 and CD40 are considered to be the intracellular TNF receptor-associated factor (TRAF) adapter proteins, which are recruited to the cytoplasmic C-terminus domains of both LMP1 and CD40 [28, 29]. LMP1 accomplishes its function by signaling through four 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)-signal transducers and activators of transcription (STAT) pathway [11]. Taken together, LMP1 is predicted to be involved in Cε gene expression by activating the NF-κB pathway and/or JAK-STAT pathway.

In conclusion, our findings suggest that Cε mRNA expression in IgE-producing cells are modulated by LMP1 gene expression and that this modulation leading to changes in expression levels of Cε mRNA might be dependent on the differentiation status of B cells upon exposure to allergen.

ACKNOWLEDGMENTS

We thank Dr Elliott D. Kieff and Dr Teruhito Yasui, Department of Microbiology and Molecular Genetics, Harvard Medical School, for providing pSG5-LMP1.

REFERENCES


