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Immunoglobulin Production Induced by EBV Reactivation: Underlying Mechanisms of Autoimmune Diseases Including Graves' Disease

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Abstract

Epstein-Barr virus (EBV) is a human herpesvirus, which latently infects B cells and is occasionally reactivated. We have been interested in EBV as an environmental factor in the development and exacerbation of Graves' disease. Serum levels of thyrotropin receptor antibodies (TRAbs), the causative antibodies of Graves' disease, are moderately correlated to EBV-early antigen (EA) antibody levels. EA antibody is a marker of EBV reactivation.

We investigated whether EBV reactivation induces TRAb production in EBV latently infected TRAb-positive B cells. Our results have demonstrated the presence of TRAb(+)EBV(+) cells in peripheral blood mononuclear cells (PBMCs) from Graves' disease patients and healthy controls. We have further confirmed the TRAb production from TRAb(+)EBV(+) cells *in vitro* when EBVs are persistently reactivated. Thus, EBV-reactivation stimulates immunoglobulin (Ig) production from host B cells.

B cells undergo germinal center reaction, and produce affinity-maturated IgG in the bone marrow for a prolonged period. Therefore, serum total-IgG level is higher than total-IgM level. In contrast, serum TRAb-IgM level is significantly higher than TRAb-IgG level, suggesting that the mechanism of TRAb-production is different from the conventional mechanism. Furthermore, TRAb-IgM level is higher in the EBV reactivation group, in which EBV antibodies are high, indicating a relation to EBV reactivation.

We induced EBV reactivation in PBMCs *in vitro* and detected the expression of activation-induced cytidine deaminase (AID) essential for the class-switch recombination of Ig, and observed every isotype of Ig-secretion in culture medium, but IgM levels were still significantly higher than IgG levels. We also detected the EBV-latent membrane protein 1 (LMP1).

We proposed the mechanism of autoantibody production. During EBV reactivation, many infectious virions are produced and infect the surrounding B cells. EBV newly-infected cells become latency 3, and express EBV-LMP1. It is known that LMP1 activates B cells polyclonally, induces anti-apoptotic effects, and promotes AID expression. Therefore, the EBV newly-infected B cells are activated and become able to be class-switched. Furthermore, EBV reactivation induces plasma cell differentiation and Ig production

Autoreactive-B cells, including TRAb-positive B cells, have difficulties in encountering their specific antigens, and thus in producing Igs. However, in the EBV reactivation state, they can produce autoantibodies, which may be the underlying mechanisms of autoimmune diseases including Graves' disease.

Keywords: Epstein-Barr virus, reactivation, autoimmune disease, Graves' disease, immunoglobulin

Graves' disease is an autoimmune-hyperthyroidism

Graves' disease accounts for 50-80% of hyperthyroidism cases (1). Thyroid hormones have many functions including sympathomimetic effects, calorigenic actions, and elevation of metabolism. Therefore, hyperthyroidism patients show heart palpitations, shortness of breath, heat intolerance, and weight loss.

The thyroid stimulating hormone (TSH: thyrotropin) receptor on the surface of thyroid follicular epithelial cells is stimulated by TSH secreted from the pituitary gland, and the follicular epithelial cells secrete thyroid hormones. However, Graves' disease patients have autoantibodies against TSH receptors (TRAbs), which bind with TSH receptors more competitively than TSH. TRAbs stimulate follicular epithelial cells to produce excessive thyroid hormones resulting in hyperthyroidism. Graves' disease is an autoimmune thyroid disease.

Hereditary factors are considered to be important in the pathogenesis of Graves' disease, because Graves' disease develops to a greater extent in siblings, and monozygotic twins show a higher concordance than dizygotic twins. Many susceptibility genes have been reported, but their relative risks do not adequately account for the pathogenesis of Graves' disease.

On the other hand, environmental factors including iodine intake, stress, and infection are also related to the development of Graves' disease. We are interested in the effects of Epstein-Barr virus (EBV) as an environmental factor.

EBV is a persistent virus that is sometimes reactivated

EBV is a human herpesvirus that was detected in the culture medium of Burkitt's lymphoma cells by Epstein *et al.* in 1964 (2). The genome of EBV is double-strand DNA of 170 kbp. EBV is the first discovered human cancer virus, and is related to the carcinogenesis of several malignant tumors including Hodgkin's lymphoma, nasopharyngeal carcinoma, and gastric cancer.

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EBV is known as the cause of infectious mononucleosis (IM). Most adults experience primary infection through saliva in their infancy, become latently infected, and show EBV antibodies in serum. The primary infection in infancy is asymptomatic, but in adolescent or after, may develop into IM, showing as a high fever, tonsillitis, lymphadenopathy, increased lymphocytes, hepato-splenomegaly, and liver dysfunction.

EBV mainly persists in B lymphocytes. When EBVs infect B cells, some B cells are lytically infected and release a lot of infectious virions when they die, but most B cells become latently infected status termed latency 3 (Table 1) (3). In latency 3, host cells express many viral antigens that could be targeted by cytotoxic T lymphocytes (CTLs). Therefore, infected cells escape from CTLs by changing their state of latency from 3 to 1 or 0 through epigenetic silencing.

Even after achieving a stable latent state, the infected cells sometimes become lytic after stimulation, which is called "reactivation" (4). Once EBV is reactivated, viral genes are replicated, and the genes that make viral particles are expressed in a cascade. Then, a lot of infectious virions are produced and released from the cells.

Table 1. Various viral antigens expressed by EBV

Reactivation		Latency 3	Latency 2	Latency 1	Latency 0
Immediate -early	BZLF1	EBERs	EBERs	EBERs	EBERs
	BRLF1	EBNA1	EBNA1	EBNA1	
Early	EA	EBNA2			
	BALF5	EBNA3			
Late	BGLF4	EBNA-LP			
	gp350/220	LMP1	LMP1		
	VCA	LMP2	LMP2		

EBV may be related to pathogenesis and/or exacerbation of Graves' disease

Several reports have suggested that serum levels of EBV antibodies are increased in some autoimmune diseases such as multiple sclerosis or systemic lupus erythematosus (2, 5).

We previously reported that EBV reactivation may be related to the development and exacerbation of Graves' disease, based on our results that serum levels of EBV-early antigen (EA) antibody and TRAb levels are moderately correlated (6). EA is a product of the EBV-BMRF1 gene expressed in the early phase of reactivation (Table 1).

B cells differentiate into antibody-producing plasma cells. It has been reported that B cell differentiation into plasma cells occurs during the same period as the reactivation of persistent EBV (7). We attribute EBV may play a role to stimulate antibody production of host B cells.

Detection of TRAb(+)EBV(+) cells

TRAbs bind to TSH receptors and stimulate thyroid follicular epithelial cells to produce excessive thyroid hormones. We hypothesized that "Reactivation of EBV persisting in TRAb-positive cells may stimulate TRAb production and secretion, and may develop or exacerbate Graves' disease", i.e., "if there are TRAb-producing B cells that are infected with EBV, the reactivation of the EBV may induce TRAb production of the host B cells" (Figure 1).

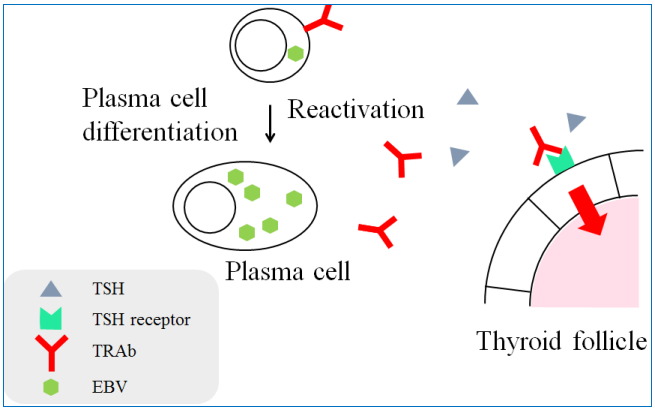

Fig. 1. Reactivation of persisting EBV in TRAb(+)EBV(+) cell stimulates TRAb production (cited from Ref. 8 with permission).

If our hypothesis was correct, there must be EBV-infected B cells that have TRAbs as the surface globulin. We investigated the presence of TRAb(+)EBV(+) cells in peripheral blood mononuclear cells (PBMCs) with flow cytometry (8).

We fluorostained EBV-encoded small RNA 1 (EBER1) by flow cytometric *in situ* hybridization to detect EBV-infected cells (9). Each EBV-infected cell had approximately 107 copies of EBER1 (2). We detected cell surface TRAbs by modifying the common radio-receptor assay system with antibodies used in measurements of serum TRAbs.

Before staining, we pre-cultured sample PBMCs to enrich EBV-infected cells, because peripheral B cells contain EBV-infected cells at a ratio of only one cell per $10^{5\text{-}6}$ cells.

We sorted the double-positive cells of EBER1 and TRAb using flow cytometry, and confirmed the presence of TRAb(+)EBV(+) cells in every samples from 8 patients and 8 controls by examination with a confocal laser microscope.

TRAb production induced by EBV reactivation

We have studied whether TRAbs are produced when EBVs persisting in TRAb(+)EBV(+) cells are reactivated *in vitro* (10). We prepared PBMCs containing TRAb(+)EBV(+) cells by pre-culturing, and transferred them from 37 ℃ to 33 ℃. The 33 ℃ culture is a physiological way to induce EBV-reactivation (10-13). It is not as efficient as reactivation using chemicals, but it is physiological and does not injure the cell membrane. We regarded the day when the cells were transferred to 33℃ culture as day 0, and collected culture fluids on day 0, 5, 10, and 12, respectively. We assayed the TRAbs level in the culture fluids and confirmed the increase of the TRAb concentration, showing TRAb production for at least one sampling point (from day 0 to day 12) in each sample. The total amount of TRAb production was greater in patients than in controls.

We prepared PBMCs cultured in 37 ℃ throughout the sampling period as the controls for 33 ℃ reactivated PBMCs. A significantly higher amount of TRAbs (days 0-12) was produced in 33 ℃ reactivated PBMCs than in 37 ℃ cultured PBMCs.

Considering that day 0 samples might be influenced by pre-culture; therefore, we calculated the sum of days 5-12, and days 10-12, and found these values to also be significantly higher in 33 °C culture PBMCs than in 37 °C culture PBMCs. These results indicated that EBV reactivation induced TRAb-production.

To confirm the reactivation induction effect of the 33 °C culture, we analyzed the cells collected on day 0 and day 12, respectively, by flow cytometry with the 72A1 antibody, a late phase marker of EBV reactivation (14, 15). When infectious virions leave their host cells in the late phase of reactivation, they use the host cell membrane with glycoprotein 350/220 (gp350/220) as their envelopes. The 72A1 antibody is an antibody against gp350/220. The number of cells in the late phase of reactivation was increased at day 12 compared to day 0. The number of TRAb(+)72A1(+) double positive cells also increased at day 12.

We sorted and observed the double positive cells, and found that the cells had abundant cytoplasm, their nuclei were not round, and red signals of TRAbs were seen inside the cells. The analysis of lymphocyte surface markers revealed that the CD79a positive B cell population increased after pre-culture, and the CD138 positive plasma cell population increased after EBV reactivation induction in the 33 °C culture. We concluded that TRAb(+)72A1(+) cells are plasma cells.

These results indicate that there are TRAb(+)EBV(+) cells in the peripheral blood of Graves' disease patients and controls, and that the EBV, persisting in the TRAb(+)EBV(+) cells, stimulates TRAb-production during EBV reactivation (Figure 1).

EBV reactivation stimulates immunoglobulin (Ig) production and activation-induced cytidine deaminase (AID) expression

Thyroid stimulating TRAbs are thought to be IgG1 class antibodies (1), but IgM class TRAbs have been reported in myxedema patients (16). We measured serum levels of total IgG, total-IgM, TRAb-IgG and TRAb-IgM in 34 Graves' disease patients and 15 controls. We found in Graves' disease patients and controls, that the serum level of TRAb-IgM is significantly higher than that of TRAb-IgG, although the serum total IgG is higher than total IgM (17). As IgG is the predominant serum Ig, we expected that level of TRAb-IgG would be higher than that of TRAb-IgM. However, the opposite results were found; serum level of TRAb-IgM was significantly higher than that of TRAb-IgG.

We measured anti-EBV antibodies in the same serum samples as our Ig and TRAb assays, and divided all data into a high anti-EBV antibody group and another group to examine the effect of EBV reactivation. The results indicated that TRAb-IgM was produced to a greater extent in the high-EBV antibody group than in another group. Thus, comparing the proportion of serum Ig with various specificities, total IgG levels were higher than total IgM levels. However, in autoantibody TRAbs, TRAb-IgM levels were higher than TRAb-IgG levels, and TRAb-IgM levels were high especially in the EBV reactivated state.

In vitro, we induced EBV reactivation in B cell-enriched pre-cultured PBMCs. During reactivation, we detected production of IgG, IgM and IgE in culture fluids with peaks on days 0 and 10 or 12 (18). We also collected culture cells and confirmed the protein expression of AID and its mRNA on day 5 and later. AID is a nucleotide-editing enzyme that is essential for class-switch recombination (CSR) of the Ig gene (19). Therefore, we suggest that production of IgG and IgE on day 10 or 12 was catalyzed by AID induced by EBV reactivation. We also observed that the production of IgM was higher than that of IgG *in vitro*.

The mechanisms of EBV-reactivation induced Ig production

As antibody-producing cells undergo CSR and affinity maturation at the germinal center, and produce affinity-maturated IgG antibodies in bone marrow for a prolonged period, IgG is widely circulated (20, 21). However, TRAbs must be produced by a different mechanism from the conventional serum Ig, because serum TRAb-IgM levels are higher than levels of TRAb-IgG.

Of the total circulating B cells, 70-90% have IgM on their surface (IgM-B cells: most of them are mature naïve B cells) (21, 22). The mechanism of TRAb production may be dependent on the proportion of B cell surface Ig isotypes in circulation. Furthermore, the production of TRAb-IgM occurs significantly in the EBV reactivation state, suggesting an association with EBV reactivation. Also in an *in vitro* experiment, the amount of total IgM production induced by EBV reactivation was higher than that of total IgG, reflecting the surface Ig isotype proportion of collected PBMCs in culture. These results suggest that the mechanism of Ig production induced by EBV-reactivation activates existing B cells non-specifically (polyclonal B cell activation) (Figure 2).

During the EBV reactivation, many infectious virions are produced and infect the surrounding B cells. Most of the newly infected cells become latency 3, and express viral antigens including the latent membrane protein 1 (LMP1) and EBV nuclear antigens (2, 3). LMP1 has many important functions. LMP1 mimics the CD40 signal and activates B cells without stimulation from specific antigens or cognate CD4 T cells (polyclonal B cell activation) (2, 23, 24). LMP1 also promotes AID expression through NF-κB up-regulation (2, 24, 25), and protects host cells from apoptosis by up-regulating bcl-2 (2).

Therefore, in EBV reactivation, LMP1 is expressed in newly infected B cells, and through LMP1 expression, polyclonal B cell activation, AID expression, and anti-apoptotic effects are induced, and then, polyclonal Ig productions are stimulated. We proposed a pathway for EBV reactivation-induced Ig production based on our results (18). Figure 2 shows the pathway steps we hypothesized from our studies.

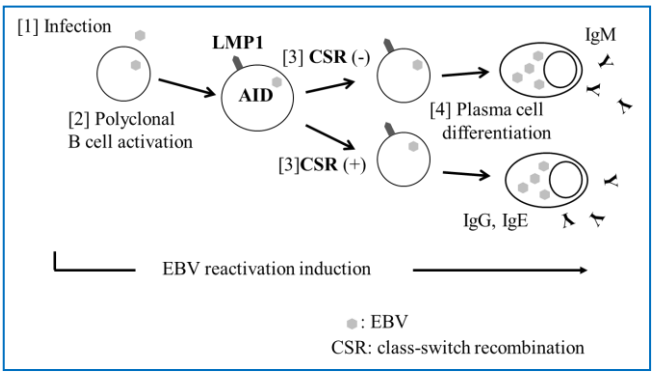

Fig. 2. The mechanisms of EBV reactivation-induced Ig production (cited from Ref. 18 with permission).

[1] B cells are infected by new EBV virions released from pre-existing plasma cells and activated B cells.
[2] Newly infected cells become latency 3, and express LMP1. LMP1 activates host cells (polyclonal B cell activation).
[3] LMP1 stimulates AID expression through NF-κB, which enables the production of class-switched Ig.
[4] EBV reactivation induces plasma cell differentiation and Ig production.

Rescue of autoreactive B cells

Circulating B cells contain autoreactive B cells that have escaped from negative selection of central immune tolerance. These autoreactive B cells have difficulty in finding their specific antigens, because self-antigens, including in the nucleus, DNA, and organelle, are usually packed inside each cell. Therefore, most autoreactive B cells are never activated, leave lymphoid tissue, and die. However, EBV-infected B cells can survive because they are transformed and immortalized by EBV.

In our model, once the infectious virions infect autoreactive IgM-B cells that have been purged from lymphoid tissues, these cells are rescued and activated polyclonally, after which they differentiate into plasma cells to produce autoantibodies that are not produced through the common pathway.

According to our mechanism of Ig production induced by EBV, various autoantibodies other than TRAbs could also be produced. This could be speculated from the fact that various autoantibodies are produced in the acute phase of IM (26, 27). Furthermore, this mechanism may explain why autoimmune diseases are often complicated by other autoimmune diseases.

Conclusions

EBV reactivation induces TRAb production from TRAb(+)EBV(+) B cells, which may develop and exacerbate Graves' disease. EBV-reactivation induces Ig production through polyclonal B cell activation, AID induction, protection from apoptosis, and differentiation into plasma cells. EBV-reactivation rescues autoreactive B cells to produce autoantibodies.

Conflict of Interest: None

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