Published in final edited form as:

Autoimmun Rev. 2009 February ; 8(4): 337. doi:10.1016/j.autrev.2008.12.008.

Aberrant Epstein–Barr viral infection in systemic lupus erythematosus[☆]

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Abstract

Serologic association, cross-reactivity of select EBV-specific antibodies with SLE autoantigens, SLE-like autoimmunity after immunization with EBV peptides, increased EB viral load in SLE patients, and SLE-specific alterations in EBV humoral and cellular immunity implicate Epstein–Barr virus (EBV) in the development of systemic lupus erythematosus (SLE). To investigate SLE-specific differences in EBV gene expression, levels of eight EBV genes were compared between SLE patients and controls by using both *ex vivo*-infected and un-manipulated peripheral blood mononuclear cells (PBMCs). Expression levels of mRNA were significantly greater by Wilcoxon signed rank test in the *ex vivo*-infected SLE patient-derived cells for 4 of 8 EBV genes, including BLLF1, 3.2-fold ($p<0.004$); LMP-2, 1.7-fold ($p<0.008$); EBNA-1, 1.7-fold ($p<0.01$); and BcRF1, a proposed DNA binding protein, 1.7-fold ($p<0.02$). The frequency of LMP-1 gene expression was significantly greater by Chi square analysis in the peripheral blood from SLE patients than controls (44% of patients, 10% of controls $p<0.05$). PBMCs from SLE patients had greater expression of latent genes as well as increased expression of both latent and lytic genes after infection, suggesting that EBV may participate in SLE etiology through several mechanisms. Such altered infection patterns may contribute to the increased levels of EBV and the molecular mimicry seen in sera from SLE patients.

Keywords

Systemic lupus erythematosus; Epstein–Barr virus; Gene expression; Latency

[☆]This work was supported by grants from the National Institutes of Health (AR48940, AR049084, RR015577, AR053483, AI007633, AR42460, AI31584, AR12253, RR020143, and RR020143), the Lou Kerr Chair in Biomedical Research at OMRF (JAJ) and Kirkland Scholar support (JBH and JAJ).

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IgA endomysium antibodies – an early predictor for celiac disease in children without villous atrophy

1. Introduction

1.1. Serologic and DNA association of EBV with SLE

Increasing evidence supports Epstein–Barr virus (EBV) infection in the etiology of multiple autoimmune diseases, such as, multiple sclerosis, rheumatoid arthritis and systemic lupus erythematosus (SLE) [1]. Examination of EBV seroconversion rates in both children and adults by multiple investigators and in diverse populations showed strong association between prior EBV infection and SLE [2–8]. Nearly 100% of SLE patients have been positive for EBV–VCA (Epstein–Barr virus viral capsid antigen) IgG responses while fewer SLE-unaffected controls have been positive for evidence of EBV infection [2–4]. The differences in infection rates observed in these studies were highly significant, with odds ratio approaching 50 in pediatric cases compared to controls ($p < 10^{-11}$) and over 10 for studies in adults ($p < 0.005$). [2–4]. Additional studies have found significant associations between SLE and IgA antibodies to EBV [5,6], and between EBV DNA and SLE [2,5,7,8]. Temporal association has also been identified between EBV and SLE (unpublished observations). Several case reports suggest that EBV infection precipitates SLE [9–11]. The high significance of the association between EBV infection and SLE makes EBV the infectious factor most strongly associated with the development of SLE.

1.2. Molecular mimicry as a mechanism for autoantibody development

Molecular mimicry is attractive as a putative mechanism to explain how EBV infection leads to lupus autoimmunity. Two EBV proteins have regions of immunologic similarity with key lupus-specific autoantigens. Some antibodies against Epstein–Barr virus nuclear antigen 1 (EBNA-1) cross-react with Sm B' [12], SmD1 [13], SmD3 [14], and 60 kD Ro [15]. Antibodies to the Epstein–Barr virus nuclear antigen 2 (EBNA-2) can also cross-react with SmD [7]. The self antigens targeted by these cross-reactive EBV antibodies are especially interesting, since the 60 kD Ro and Sm B' antigens have key roles in SLE autoantibody development. Ro autoantibodies are among the first to appear in SLE [16]. Antibodies against Sm are found almost exclusively in lupus patient sera, to such an extent, that they are considered a classification criterion [17] and usually develop close to the time of SLE classification [16].

The earliest detectable epitopes bound by autoantibodies against either 60 kD Ro or Sm B' are cross-reactive with the EBV antigen EBNA-1 [15,18,19]. Immunization of mice and rabbits with EBNA-1 sequences corresponding to these cross-reactive regions leads to epitope spreading and lupus-like autoimmunity [12,15,20]. Immunization of mice with full-length EBNA-1 DNA elicits the production of IgG antibodies to Sm and double-stranded DNA (dsDNA) suggesting that the anti-Sm response occurs as a consequence of antigenic cross-reactivity through EBNA-1 antibodies [21]. These animal models demonstrate that cross-reactive EBV immunity is capable of inducing autoimmunity *in vivo* through epitope spreading.

1.3. SLE-specific gene expression differences

One obstacle to establishing EBV as a causative agent for SLE is that very few people will develop SLE, while more than 90% of adults are infected with EBV [3]. EBV does not naturally infect other species, impeding work that would otherwise proceed in animal models. Work has therefore focused on EBV infection and immunity in SLE patients.

Results of these studies demonstrated that EBV infection has unique features in lupus patients. Compared to EBV-positive, SLE-unaffected controls, SLE patients maintain up to a 40-fold increase in copy number of EBV DNA [22] and increased numbers of EBV-infected cells in the periphery [23]. The higher level of EBV in SLE patient peripheral blood suggests a potential failure to suppress EBV infection as a characteristic feature of SLE.

1.4. Abnormal T cell immune responses to EBV in SLE compared to controls

As reviewed by Kang, et al. [22], CD4+ T cells play a crucial role in promoting an antiviral response and maintenance of virus-specific CD8+ T cells. However, in the absence of cytotoxic T cells, CD4+ T cell-mediated cytotoxicity is important for control of EBV infection. Evidence for a selective dysregulation of EBNA1-specific T cell responses, such as disrupted antibody-dependent cellular cytotoxicity of EBV-infected cells and defective T cell suppression of EBV-induced B cell proliferation, have been associated with lupus [24–26]. SLE patients compared to matched controls show decreased CD8+ T cell cytotoxicity [27], but increased CD4+ T cell recognition of EBV [22]. These results suggest that SLE patients have defective control of latent EBV infection arising from decreased CD8+ T cell responses. Compensatory increased CD4+ T cell responses which are directed against a broader array of EBNA-1 targets may also increase the probability of SLE patient T cells providing help to generate the cross-reactive humoral responses which are found in pediatric SLE patients.

1.5. Abnormal humoral immune responses to EBNA-1 in SLE compared to controls

The humoral response to EBNA-1 is significantly altered in SLE patients. Antibodies against EBNA-1 are more frequently detected in lupus patients than in controls, and the pattern of epitopes bound by antibodies is more diverse in SLE patients [26]. Anti-EBNA-1 antibodies from control individuals recognize primarily a large glycine–alanine repeat region of EBNA-1, while the antibodies from lupus patients recognize epitopes distributed over the entire protein [26]. Antibodies against cross-reactive motifs found in EBV proteins are commonly found in SLE patient sera but are generally not found in the sera of controls [28–30]. This broad, potentially cross-reactive immune response in SLE patients indicates a loss of tolerance to cross-reactive antigens and appears to be the starting point for molecular mimicry.

1.6. EBV gene expression and SLE

Long-term EBV-transformed cells express a type of latency characterized by the expression of multiple latency-associated genes [31]. In human infection, EBV infection normally progresses to latency, mainly persisting in memory B cells that have extremely limited or absent viral gene expression [32]. Examination of EBV gene expression in the peripheral blood from SLE patients revealed increased gene expression; the viral genes BZLF1, LMP-1, LMP-2 and EBNA-1 were expressed more frequently in patients than in controls [23].

Alterations in the process or maintenance of EBV infection in individuals susceptible to lupus could promote the development of autoimmunity through multiple mechanisms. Episodes of virus reactivation, as detected by increases in viral DNA, may correlate with disease activity, such as seen in multiple sclerosis [33]. Higher levels of lytic infection could maintain the immune system in a more activated state, as well as increasing the numbers of infected B cells and providing increased antigen leading to a more intense immune response. Higher expression of latent proteins may affect apoptosis and cell activation [1]. An increased level of EBNA-1 expression or increased antigen presentation of EBNA-1 to B cells in lupus-prone individuals may explain how lupus-specific differences in the humoral response to EBNA-1 are generated.

Since early infection and established latency represent different B cell and viral states, it is important to understand gene expression differences in each state. To detect potential lupus specific differences in EBV gene expression, we measured EBV gene expression in freshly EBV-infected peripheral blood mononuclear cells (PBMCs) from lupus patients and unrelated controls.

2. Materials and methods

2.1. Patients and cells

Ten SLE patients from clinical practice and 10 age-, race-, and sex-matched controls were recruited for the study. The average age was 42.125 ± 10.8 years for the patients and 42.35 ± 9.75 years for the controls. Five of the patients were being treated with hydroxychloroquine, 7 with prednisone, 4 with azathioprine, and 1 with methotrexate. The study included 8 European-American, 1 African American, and 1 American Indian pairs. Nine of the matched pairs were female. Informed consent for the studies was obtained from each participant in accordance with the protocol as approved by the institutional review board at the Oklahoma Medical Research Foundation. All patients met the revised American College of Rheumatology classification criteria for SLE [17].

PBMCs were isolated from all participants, purified by density gradient centrifugation with lymphocyte separation medium (Mediatech, Herndon VA), and infected with EBV. Twelve days post-infection, RNA was extracted and analyzed for EBV gene expression by real-time PCR. Eight EBV genes were selected for analysis (Table 1).

2.2. Ex vivo EBV infection

For infection experiments, the B95.8 strain of Epstein-Barr virus was incubated with 1×10^6 PBMCs per ml in 1:1 (B95-8 supernatant: RPMI 1640 with 10% FBS). One $\mu\text{g/ml}$ cyclosporin A was added to cultured cells. Infected PBMCs were incubated at 37°C , 5% CO_2 for 12 days. Twelve days was selected as the endpoint since cells are at an early blast stage but lytic cycle genes are still expressed at high levels. For B-cell experiments, blast-like morphology was confirmed by the preponderance of clusters of large, non-adherent cells by microscopy.

2.3. Real-time PCR

RNA was extracted from PBMCs with Trizol reagent (Invitrogen, Carlsbad, CA). cDNA was generated from 5 μg DNase-treated RNA using the Superscript First Strand Synthesis kit (Invitrogen), with random hexamers as primers, according to the manufacturer's directions. Each primer pair was amplified in duplicate by real-time PCR using cDNA from 125 ng of RNA with Syber Green Master Mix (Applied Biosystems, Foster, CA) on the ABI Prism 7700 Sequence Detector (Applied Biosystems). Primer sequences are listed in Table 1. Melting curve analysis was performed with single peaks observed for each primer pair, and no amplification was seen when cDNA from the EBV-negative cell line Ramos was used as template. The $\Delta\Delta\text{Ct}$ method was validated for these primers by comparing the results of serial dilutions as previously described [34].

2.4. Statistical analysis

Results were analyzed by using the Wilcoxon signed-rank test for paired samples and the Chi-square test to evaluate differences in prevalence. *P* values of less than 0.05 were considered significant.

3. Results and discussion

Expression levels of 7 out of 8 genes were greater by an average of 1.9-fold in SLE patients compared to matched controls (Fig. 1A). The increase in gene expression was significant in 4 EBV genes by the Wilcoxon signed-rank test: BLLF1 with a 3.2-fold increase ($p=0.003$); LMP-2 with a 1.7-fold increase ($p=0.0079$); EBNA-1 with a 1.7-fold increase ($p=0.0098$); and BCRF1 with a 1.7-fold increase ($p<0.02$) (Fig. 1A). The viral functions of these gene products are provided (Table 1). Two of the significantly upregulated genes are normally expressed during lytic infection, and two are found in latent infection. Given this distribution, the data

do not suggest that there is either a specific enrichment in latent or lytic infection in cells from SLE patients. Rather, they point to a broader representation of EBV genes in lupus patients compared to matched controls.

To characterize lupus-specific gene expression patterns of persistent EBV infection, PBMCs from lupus patients and matched controls were tested for EBV gene expression without the addition of endogenous virus. RNA was isolated from 6 patients and 9 controls, and real-time RT-PCR was performed to quantify expression of BLLF1, BCRF1, BZLF1, EBNA-1, LMP-1, LMP-2, EBER-1, and EBNA-LP.

Gene expression was more commonly detected in SLE patients than in the controls. The lytic cycle gene BLLF1 was found in 66.7% of patients and 44% of controls, and BCRF1 was found in 16.7% of patients and 11.1% of controls. BZLF1 was not detected. EBNA-1 and EBNA-LP RNA were detected in 66.7% of patients and 33.3% of controls. EBER-1 was found in 83% of patients and 55.6% of controls. The most drastic difference in EBV gene expression in de novo cells was LMP-1 (Chi-square analysis, $p < 0.038$, 50% of patients and 11% of controls). LMP-2 expression was found only in PMBCs from one control (Fig.1B). The frequency of latently infected B cells in the peripheral blood is reported to be extremely low, on the order of 1–100 per million cells [22,23,35]. This low level of expression suggests that the presence of previously infected B cells should have minimal effects on the results of the *ex vivo* infection.

This study was initiated to investigate lupus-specific differences in EBV gene expression at different stages of the viral cycle. The selection of genes that were studied included genes that were expressed in both lytic and latent infection and included transcription factors, structural proteins, latency-promoting factors and B cell regulatory proteins. Surprisingly, almost all of the EBV genes studied trended towards greater expression levels in PBMCs from the lupus patients than from the controls regardless of infection status (lytic or latent). The four EBV genes BLLF1, LMP-2, EBNA-1 and BCRF1 that had significantly higher expression during early infection in the lupus patients have the potential to influence the development of lupus.

EBNA-1 is the most likely protein to be involved in molecular mimicry for multiple reasons. This protein has multiple regions of immunologic similarity with lupus-associated autoantigens [15]. Lupus patients have an altered humoral immune response to EBNA-1 [15]. Animals immunized with these cross-reactive sequences develop lupus autoimmunity and clinical disease [12,20]. We have previously reported that lupus patients have autoantibodies that bind specific regions of EBNA-1. Additionally, rabbits immunized with a short EBNA-1 peptide mount an immune response to the peptide of immunization as well as develop antibodies that cross-react with the short peptide of Sm B/B' [2]. High expression of viral EBNA-1 in a host would be likely to increase its availability to B and T cells, increasing potential for the formation of cross-reactive antibodies and more antigenic stimulation to drive the response towards autoimmunity. LMP-2 influences the development and signaling of B cells. B cells are responsible for producing the autoantibodies that are at the foundation of lupus, dysregulation of these cells may have dramatic consequences. Additionally, overexpression of the LMP-2B form of LMP-2 leads to increased switching to lytic infection, potentially destabilizing the normal pattern of latency [36]. BLLF1 and BCRF1 are expressed during lytic infection, and the increase in expression of these genes suggests that viral load and replication are also greater in lupus patients. Greater lytic activity would influence antigen availability, inflammation, and drive the immune system towards activation rather than tolerance.

LMP-1 RNA was detected significantly more frequently in the peripheral blood from SLE patients than from controls. LMP-1, in addition to its normal B-cell survival function, can stimulate production of B-cell activating factor belonging to the TNF family (BAFF) [37], which may aid in breaking self-tolerance [1,38]. BAFF levels are known to be elevated in SLE

[39] and may even correlate with disease activity. All of these pathways may influence the development of SLE in response to EBV infection of susceptible individuals, and warrant further investigation.

In a previously reported study [23], BZLF1 expression was detectable in RNA from lupus patients but not from controls. From this finding it was suggested that lupus patients have greater lytic infection in the peripheral blood than controls where lytic infection is extremely rare. In freshly isolated cells, we did not detect BZLF1 expression, however, we did find BLLF1 and BCRF1 expression in both patient and control samples. In concordance with the study published by Gross et al [23], the proportion of samples with detectable levels of lytic transcripts trended higher in the patients than in the controls, agreeing with the idea that lytic infection may be more prevalent in lupus patients.

Gross and colleagues suggested that the altered presentation and biology of EBV infection in lupus patients is due to a modification in the types of circulating B cells, specifically a dysregulation in the homeostasis of memory B cells [23]. More recent data have led to the conclusion that EBV occupies a skewed niche within the memory compartment and that EBV-infected cells accumulate more somatic hypermutations in immunoglobulin genes [40]. Although our results could also be explained by altered B cell patterns in the peripheral blood of patients, this does not prevent EBV from being implicated in lupus. Instead, it identifies a cellular mechanism, perhaps dependent on genetics of the individual with lupus susceptibility, whereby EBV infection could lead to increased gene expression, viral load, and altered immunity promoting the development of lupus. Altered expression of a variety of EBV genes has the potential to disrupt the normal balance between EBV activity and the immune system. Increased viral transcription is a feasible explanation for many of the lupus-specific alterations in EBV immunity.

Take-home messages

- EBV infection is a credible etiologic agent for aspects of systemic lupus erythematosus.
- Compared to controls, SLE patients have broader EBV gene expression, increased expression levels of particular genes, and altered anti-EBV immunity.
- Both latent and lytic EBV genes are overexpressed in SLE patients.
- Less effective suppression of EBV infection has the potential to contribute toward lupus development.

This study was designed in order to evaluate possible differences between children with ant-endomysium antibodies (EMA) positivity and normal small bowel mucosa and children with positive EMA and an enteropathy diagnosed as celiac disease (CD). **Grodzinsky E. et al. (Acta Paediatrica 2008; 97: 972-6).** Children with suspected CD and positive EM ($\geq 1/10$) undergoing small bowel biopsy during 1996 to 2002, were investigated (n = 133). Data registered were: year and month of birth, timing of the first biopsy, sex, heredity for CD, dermatitis herpetiformis and diabetes mellitus and outcome of the anti-gliadin antibody test (AGA). The case group, with EMA positivity and normal histology (n = 39; 59% female, mean age at the first biopsy 7.3 years, range 1.4-16), was compared with the disease control group, with positive EMA and a biopsy suggestive and further on diagnosed as CD (n=94; 56% female; mean age 7.6 years at the first biopsy, range 0.70-17). AGA positivity and heredity for CD were found to predict the outcome of a pathological jejunal mucosa. Nineteen of the 39 children in the case group were re-biopsied of whom 11 had developed an enteropathy during a follow-up period of 2-7 years. Thus, EMA positivity in

the absence of small bowel enteropathy could be a very early predictor for later overt CD, and necessitates further follow-up, especially if the child is AGA positive and there is a family history of CD.

Acknowledgments

We thank the NIH, Kirkland Scholars Program, and the Lou Kerr Chair in Biomedical Research for funding this work. We thank Kristina Wasson-Blader, PhD for review and revision of the manuscript.

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Fig.1.

EBV gene expression in SLE patients and controls. EBV gene expression was analyzed by real-time PCR. A. Significant increases in expression of 4 genes were found in 12-day cultures of EBV-infected cells from SLE patients compared to EBV-infected cells from controls ($n=10$ pairs). Gene expression is shown in terms of mean fold increase in the patient samples compared to controls. Error bars indicate standard error. B. Both lytic (BLLF1 and BCRF1) and latent (EBER 1, EBNA-1, LMP-1, and LMP-2) gene expression was detected in PBMCs from both patients and controls immediately after isolation. LMP-1 expression was significantly greater in freshly isolated PBMCs from patients than from controls ($p<0.05$, $n=6$ patients, 9 controls).

Table 1

EBV genes studied

Gene	Lytic/latent	Function	Primers used for analysis
BLLF1	Lytic	Major glycoprotein gp350/220	CATTGGTAGCCGTTCTGTGTGATAAT GCGAGCAATCGGACATTTGACAT
BcRF1	Lytic	DNA binding protein	AGGGCCTGTTTGATTTCTCCAAGAG TTTGTACGTTCCGCCGAATGTC
BZLF1	N/A	Transcription factor for switch to lytic phase	CGCCTGGGCATTTCGTGTTAGTATAT AAGAATCGGGTGGCTTCCAGAA
EBNA-1	Latent	Transcription factor, links viral DNA to chromosomes	CTTGTTATGACAAAGCCCGCTCC TGGAACCAGGGAGGCAAATCTAC
EBNA-LP	Latent	EBNA leader peptide	CTCCCGCTCGGTCTCTTAGAGAGT TTGGCGCTGGGTGGTTACTGT
LMP-1	Latent	Oncogene, CD 40 homolog	TCATCGTGGTGGTGTTCATCACTG ATCTCCTTTGGCTCCTCCTGTTTC
LMP-2	Latent	Interferes with stimulatory signals	TGATTGTCGCTGGCATACTCTTCAT CAGGCCACCGAGGCACATAA
EBER-1	Latent	Interferes with IFN response	CCTACGCTGCCCTAGAGGTTTTG CCTCACCACCCGGGACTTGTAC