Med Microbiol Immunol (2005) 194: 115–120
DOI 10.1007/s00430-004-0230-5

ORIGINAL INVESTIGATION

Shan-Fu Yu · Huei-Chuin Wu · Wen-Chan Tsai
Jeng-Hsien Yen · Wen Chiang · Chung-Yee Yuo
Sheng-Nan Lu · Lien-Chai Chiang · Chung-Jen Chen

Detecting Epstein-Barr virus DNA from peripheral blood mononuclear cells in adult patients with systemic lupus erythematosus in Taiwan

Received: 17 November 2003 / Published online: 17 September 2004
© Springer-Verlag 2004

Abstract Epstein-Barr virus (EBV) has been found by many serology studies to be associated with systemic lupus erythematosus (SLE). However, the results of DNA studies have been conflicting. Therefore, instead of antibody to EBV, we studied the association between EBV DNA and SLE. In this case-control study in Taiwan, we enrolled 87 SLE patients and 174 age- and sex-matched controls. Peripheral blood mononuclear cells of SLE patients and matched controls were tested for EBV DNA by polymerase chain reaction (PCR) and Southern blot. Of the 87 SLE patients, 71 (81.6%) were found to be positive for EBV DNA, while 85 (48.9%) of the 174 controls (odds ratio 4.64, 95% confidence interval 2.50–8.62, $P < 0.0001$) were positive. While the EBV DNA-positive rate did not decline with age in SLE patients ($P > 0.05$), it did decline with age in controls ($P < 0.05$). Furthermore, based on a real-time quantitative PCR study, we have found a significant difference between EBV viral load in SLE and controls ($P = 0.008$). Therefore, in our molecular study of DNA level, we found evidence for the association of EBV infection and SLE, suggesting that EBV contributes, if not to the development of SLE, then to disease perpetuation.

Keywords Systemic lupus erythematosus · Epstein-Barr virus · DNA

C.-Y. Yuo
Department of Biomedical Science and Environmental Biology, Kaohsiung Medical University, Kaohsiung, Taiwan

H.-C. Wu
Institute of Medicine, Kaohsiung Medical University, Kaohsiung, Taiwan

W.-C. Tsai · J.-H. Yen
Department of Internal Medicine, Kaohsiung Medical University, Kaohsiung, Taiwan

L.-C. Chiang
Department of microbiology, Kaohsiung Medical University, Kaohsiung, Taiwan

S.-F. Yu · S.-N. Lu
Department of Internal Medicine, Kaohsiung Chang Gung Memorial Hospital, Kaohsiung, Taiwan

W. Chiang
Department of Clinical Laboratory, Kaohsiung Medical University, Kaohsiung, Taiwan

C.-J. Chen (✉)
Division of Rheumatology, Allergy and Immunology, Department of Internal Medicine, Kaohsiung Chang Gung Memorial Hospital, No. 123, Ta-Pei Road, Niao-Sung Hsiang, 833 Kaohsiung Hsein, Taiwan
E-mail: chungjen@adm.cgmh.org.tw
Tel.: +886-7-7317123 ext. 8800
Fax: +886-7-7322402

Introduction

Systemic lupus erythematosus (SLE) is a chronic inflammatory autoimmune disease, characterized by the production of large amounts of autoantibodies, including antinuclear (ANA), anti-double stranded DNA (anti-dsDNA), anti-Sm, anti-nuclear RNP and anti-phospholipid antibodies [5]. Antibodies against the spliceosome, also referred to as anti-Sm and anti-nRNP, are common in lupus and can be found in about 30–50% of patients [6]. This major autoimmune response targets primarily the Sm B/B' protein [1]. The epitope peptide PPPGMRPP, which induces the earliest anti-Sm autoimmune humoral response, has been found to have the same structure as the Epstein-Barr nuclear antigen-1 (EBNA-1) peptide PPPGRRP [6, 9, 11]. A high degree of homology has also been demonstrated between the EBNA-2 sequence and the antigenic Sm-D1 [8]. This molecular mimicry may play an important role in the induction of anti-Sm B/B' and anti-Sm D1 by Epstein-Barr virus (EBV) infection in SLE patients. Therefore, many investigators have suspected that EBV might critically be involved in the etiology of SLE.

The first positive link between EBV infection and SLE was found when a higher titer of anti-EBV antibody was noted in SLE in 1971 [3]. Since then, many studies have used various angles to investigate the possibility of this link, many with different conclusions [4, 14, 21, 24, 26]. Twenty-six years later, in 1997, James et al. [10] performed a large-scale case-control study to look at the infectivity rate in children and teenagers with SLE and found a striking association between EBV seroconversion and SLE with an impressive odds ratio of 49.9. Then, using the same method, James et al. [12] extended this study to adult lupus patients and found an odds ratio of 9.35. However, the increase in antibodies in SLE was thought by some to be brought about by generalized immune hyper-reactivity in lupus rather than by any specific property of the EBV [21]. Thereafter, it has been thought that the best way to clarify this question would be at the DNA level. Since previous EBV DNA studies in SLE were inadequate due to sample size, and since their results conflicted with each other [8, 10, 15, 24], we conducted this strict case-control study using a larger number of cases and a sensitive method of detecting EBV, i.e., qualitative and quantitative assay of EBV DNA.

Materials and methods

Patients and controls

For the case-control study, 87 patients who satisfied the 1997 updating American College of Rheumatology (ACR) revised criteria for the classification of SLE were enrolled [7]. As normal controls, 174 individuals, who did not report having SLE, were matched for age and sex. The mean age of the SLE patients was 35.3 ± 9.7 years and the normal controls 34.8 ± 10.0 years ($P = 0.575$, by t-test). The age range in both groups was 20–64 years. The female to male ratio was the same between SLE and healthy control (80 females and 7 males in the SLE group, 160 females and 14 males in the control group). All the SLE patients and controls were Asian.

Isolation of peripheral blood mononuclear cells

Peripheral blood (2 ml) was centrifuged in an EDTA tube at 2,500 rpm for 10 min. After removing the plasma, peripheral blood mononuclear cells (PBMCs) were harvested by 2 ml Ficoll-Paque plus (Amersham Biosciences) at 1,300 rpm for 20 min. The mononuclear cell layer was carefully aspirated and washed in phosphate-buffered saline (PBS) at 1,100 rpm for 10 min.

DNA extraction from PBMC

The cell pellet was resuspended in 100 µl PBS. Then, 800 µl solution I (25 mM EDTA, pH 8.0; 75 mM NaCl) was added and mixed with 80 µl 10% SDS and 10 µl proteinase K in a 50°C water bath for 3 h. DNA was extracted using a standard phenol-chloroform method and precipitated with ethanol. The concentration of DNA was determined by spectrophotometrically at 260 nm. Genomic DNA was stored at the concentration of 500 ng/µl.

Detection of EBV DNA by PCR/Southern blot

PCR

PBMC DNA (500 ng) was applied to detect the EBV genomes by PCR using the primers: forward 5’-CCAGAGGTAAGTGGACTT-3’ and reverse 5’-GACCGGTGCCTTCTTAG-3’ [10]. The PCR product of 122 nucleotides was sequenced and shown to be identical to one published previously (position 14,614–14,735, GenBank accession no. V01555). PCR reactions contained, in total 50 µl, 4 µl template DNA, 5 µl 10x reaction buffer (50 mM KCl, 10 mM TRIS-HCl at pH 8.0, 1.5 mM $MgCl_2$, 0.1% Triton X-100, from Protech Technology), 0.2 mM each of dNTP, 0.5 µM primers and 2 U Taq DNA polymerase. PCR was performed in the thermal cycler, GeneAmp 2400 (Perkin-Elmer, Branchburg, NJ). The cycles used were as follows: 2 min at 95°C, 1 min at 59°C, 1 min at 72°C (2 cycles), 2 min at 94°C, 1 min at 58°C, 1 min at 72°C (2 cycles), 1 min at 94°C, 1 min at 57°C, 45 s at 72°C (35 cycles) and extension for 5 min at 72°C.

Southern blot

PCR products were Southern-blotted with a digoxigenen (DIG)-labeled probe corresponding to positions 14,639–14,676 [10]. The probe labeling with DIG-dUTP/dATP was performed with 100 pmol oligonucleotides, 50 U terminal transferase, 1 µl DIG-dUTP and dATP, 4 µl reaction buffer and $COCl_2$ solution in a final volume of 20 µl. The detailed procedure for Southern blotting is showed below.

Transfer and fixation of PCR product PCR product (20 µl) was loaded onto a 2% agarose gel, and run in the 0.5× TBE buffer for 30 min at 100 V. The gel was rinsed with 0.25 M HCl for 4 min, denaturation solution (1.5 M NaCl, 0.5 M NaOH) for 3 min, and neutralizing solution (1 M TRIS, 2 M NaCl, pH 5.0) for 3 min. Meanwhile, a positive-charged nylon membrane was briefly pretreated with 6× SSC. The gel was transferred onto the pretreated nylon membrane in 20× SSC transfer buffer. Positive pressure of 40-cm H_2O was applied for 1 h to facilitate transfer. The membrane was air dried. DNA fixation was performed using 120 mJ in a UV-cross-linking machine (Startalinker, Stratagene, La Jolla, CA).

Hybridization The transferred membrane was prehybridized in the hybridization buffer, DIG Easy

Hyb (Roche Diagnostics, Germany) for 1 h at 42°C. DIG-tailed probe (10 pmol/ml hybridization buffer) was added. This mixture was incubated with the membrane overnight at 42°C. After hybridization, the membrane was washed twice with wash buffer I (2× SSC, 0.1% SDS) for 5 min, and twice with wash buffer II (2× SSC, 0.1% SDS) for 15 min at 65°C. The membrane was then rinsed in maleic acid buffer (Roche Diagnostics) for 2 min.

Detection To visualize probe-target hybrids, chemiluminescence detection was performed as described in the DIG Wash and Block Buffer Set and DIG Chemiluminescence Detection kit (Roche Diagnostics). First, the membrane was blocked with Blocking Solution for 30 min and the probe-target hybrids were localized with 1:1,000 dilution of anti-DIG AP antibody solution. Unbound antibody was then washed off twice with wash buffer. The chemiluminescent substrate, CSPD was added to the blot and the damp membrane was incubated for 10 min at 37°C. Finally, the membrane was sealed in the envelope and exposed to X-ray film for about 20 min.

The detection limit for the PCR/Southern blot is 9 viral copies in 1 μg DNA (5 viral copies in 10^5 PBMC).

Quantification of EBV viral load using a LightCycler PCR

The quantification of EBV viral load was performed using a LightCycler-EBV Quantification Kit (Roche Molecular Biochemicals, Mannheim, Germany). Total reaction volume of 20 μl contained 15 μl master mix and 100 ng of the corresponding DNA template. Reaction capillaries (Roche Diagnostics) were loaded and centrifuged before being placed in the LightCycler instrument (Roche Applied Science). PCR amplification was performed as follows: 10 min of denaturation at 95°C followed by 45 cycles of 10 s at 95°C, 15 s at 55°C, and 15 s at 72°C for amplification, 60 s at 40°C for the melting curve, and 30 s at 40°C for the cooling step.

Statistics

Fisher's exact test was used to analyze the differences in frequencies of positive EBV DNA in PBMC between patients with SLE and healthy controls. Chi-square test for the linear trends was measured to compare the relationship between age in the two different groups. Mann-Whitney U test was used to compare EBV viral load. A P value of less than 0.05 was regarded as statistically significant. The odds ratio with 95% confidence interval (CI) was calculated to estimate the risks for acquiring SLE when PBMC are found positive for EBV DNA. All statistical analyses were performed using SPSS software (version 10.0.1).

Results

Of the 87 SLE cases tested, 71 (81.6%) had detectable EBV DNA. Of the 174 matched controls, 85 (48.9%) had detectable EBV DNA (odds ratio 4.64, 95% CI 2.50–8.62, $P < 0.0001$; Table 1).

In the control group, the positive rate of EBV DNA in PBMC declined with age ($P < 0.05$, chi-square test for the linear trends). In the SLE group, the frequency of positive EBV DNA remained at 74.2% or more regardless of age ($P > 0.05$, chi-square test for the linear trends) (Fig. 1).

EBV viral load was determined for 21 cases with SLE and 21 matched controls. Patients with SLE had a higher number of copies of EBV DNA in PBMCs than healthy controls (means ± SEM, 216.2 ± 60.5 vs 44.6 ± 19 copies/μg DNA, respectively; $P = 0.008$, by Mann-Whitney U test) (Fig. 2).

Discussion

This study has provided definite molecular DNA evidence associating EBV infection and SLE. There have been four previous studies using detection of EBV DNA to determine whether there was a relationship between EBV virus and SLE [8, 10, 15, 24] (Table 2). Two studies produced positive results, two negative results. We therefore decided to test for EBV DNA in PBMC and SLE in a case-control study with a large sample size.

We used PCR/Southern blot to detect EBV DNA, a method known to be more sensitive and more specific than simple PCR assay. The detection limit of this PCR/Southern blot assay is 9 viral copies in 1 μg DNA (5

Table 1 Detection of EBV-DNA from PBMC in cases of SLE patients and matched controls (*EBV* Epstein-Barr virus, *PBMC* peripheral blood mononuclear cells, *SLE* systemic lupus erythematosus, *O.R.* odds ratio, *CI* confidence intervals)

Ages (years)	SLE patients, no. positive/total tested (%)	Normal controls, no. positive/total tested (%)	O.R.	95% CI of O.R.	*P* value
20–29	23/31 (74.2)	40/62 (64.5)	1.58	0.16–4.12	0.242
30–39	19/25 (76)	22/50 (44)	4.03	1.38–11.8	0.008*
40–49	25/26 (96.2)	22/52 (42.3)	34.1	4.29–271.01	< 0.0001*
> 50	4/5 (80)	1/10 (10)	36.0	1.77–732.13	0.017*
Total	71/87 (81.6)	85/174 (48.9)	4.64	2.50–8.62	< 0.0001*

*$P < 0.05$ indicates significance by Fisher's exact test

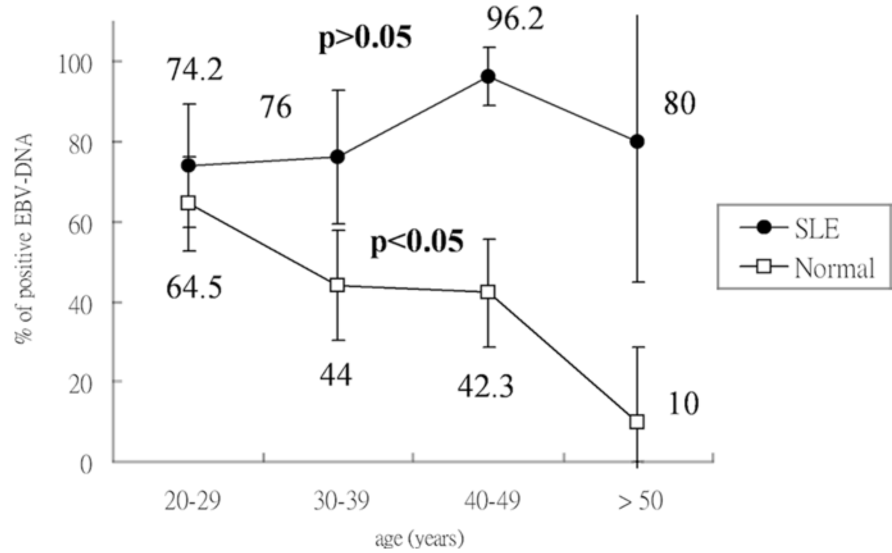

Fig. 1 The relationship of positive rate of EBV DNA with age between SLE patients and normal controls. In the SLE group, positivity for EBV DNA persists at high levels. In normal controls, the EBV DNA-positive rate declines with increasing age. $P<0.05$ value indicates significance (chi-square test for the linear trends). *Bars* indicate 95% confidence intervals for the % of cases positive for EBV DNA (*EBV* Epstein-Barr virus, *SLE* systemic lupus erythematosus)

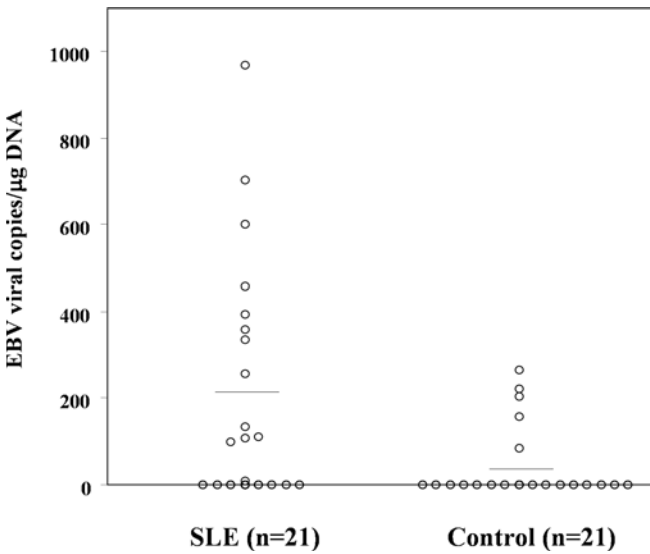

Fig. 2 EBV viral copies/μg DNA in peripheral blood mononuclear cells are compared between SLE patients ($n=21$) and healthy controls ($n=21$); $P=0.008$ (Mann-Whitney U test between two groups). *Horizontal bar* indicates mean copies of EBV DNA

viral copies in 10^5 PBMC). Therefore, we believe that this method is sufficiently sensitive. The EBV DNA-positive rate in SLE was higher than in normal control, with an odds ratio of 4.64 using this qualitative assay (Table 1). Our viral load study demonstrated that patients with SLE had a 4.8-fold increase in EBV genome levels compared with the control group (Fig. 2). Recently, Kang et al. [13] described a similar finding, with SLE patients showing a 40-fold increase in EBV viral load in PBMC compared with health controls. Taken together, an association between EBV DNA and SLE has been established.

Another question is whether EBV is the cause or the result of SLE. EBV may contribute, at least in part, to development of lupus. After primary EBV infection, EBV resides and remains in human B cells [19]. These B cells are central to the production of autoantibodies, which are critical for the laboratory diagnosis and clinical manifestation of SLE [20]. EBV nuclear antigen-1 and –2 (peptide sequences PPPGRRP and GRGRGRGR) share highly similar structures with two lupus autoantigens, Sm B/B' and Sm D1, respectively [1, 8, 9, 11, 18, 23]. In the lytic cycle, EBV expresses a protein, Zta, which serves a critical function in the transcription transactivator of the human IL-10 [17], which is known to be able to

Table 2 EBV-DNA-positive rates in SLE patients and normal control. Age is given in years (*??* not recorded, *P* PCR, *P + S* PCR/Southern blot)

Author	Specimen source	Age group of SLE (control)	Method	SLE, no. positive/total tested (%)	Controls, no. positive/total tested (%)	*P* value
Tsai et al. [24], Taiwan	PBMC	13.0 ± 8.1 (16.9 ± 3.3)	P + S	3/20 (15)	0/20 (0)	0.231
James et al. [10], USA	PBMC	15.79 ± 2.15 (15.4 ± 2.51)	P	32/32 (100)	23/32 (72)	0.002*
Lau et al. [15], Hong Kong	PBMC	??	P + S	20/34 (59)	16/22 (73)	0.394
Incaprera et al. [8], Italy	Oropharyngeal lavage fluid	??	P	8/15 (56)	6/28 (21)	0.046*
Present study, Taiwan	PBMC	35.3 ± 9.7 (34.8 ± 10.0)	P + S	71/87 (81.6)	85/174 (48.9)	< 0.0001*

*$P<0.05$ indicates significance by Fisher's exact test

increase anti-dsDNA production and reduce the number of cytotoxic T cells [16]. Taken together, these findings suggest that EBV may play an role in the development of SLE.

From a different viewpoint, SLE patients may have an immune defect in the management of EBV infections. Aya et al. [2], for example, found antibody-dependent cellular cytotoxicity for EBV-infected lymphocytes to be significantly more impaired in SLE patients than in normal controls. Cytotoxic T cell-mediated immunity is required to control EBV infection and kill EBV-infected B cells. Tsokos et al. [25] also found that SLE patients have defective suppressor T cell responses to EBV induction of normal B cell response. In addition, SLE had increased EBV-specific $CD4^{+}$ T cell response and perhaps decreased EBV-specific $CD8^{+}$ T cell response. This altered T cell response against EBV virus means that SLE patients have defective control of latent infection [13]. The primary EBV infection, which occurs in most individuals during childhood or adolescence, causes latent or lifelong infection in the host. As shown in Fig. 1, EBV DNA-positive rate declined with age in normal groups. However, positive rate for EBV DNA did not decline in SLE, and remained at between 74.2% and 96.2% in these patients, regardless of age, suggesting that SLE patients indeed do have some defect that prevents them from controlling the EBV infection.

More than any of the conflicting studies over the past 30 years, this study provides molecular DNA evidence associating EBV infection with SLE, although the exact mechanism for this association remains unclear. Further studies are needed to investigate the relationship of EBV infection to the development of SLE or its disease perpetuation.

Acknowledgements We are very grateful to our patients for donation of blood samples. The technical assistance of Miss Pei-Pei Chen, Miss Ming-Chi Yang and Mr. Hang-Ming Chang is highly appreciated. We thank Mr. Steed for editorial assistance with this manuscript. This study was supported by the Yu-Zu memorial prize for lupus research, Kaohsiung Medical University (grant no. KMU 92-A-03), and Chang Gung Memorial Hospital (grant no. BMRP 743). We are grateful for the encouragement and guidance from Prof. Kuei-Hsiang Lin and Prof. Hong-Wen Liu at Kaohsiung Medical University.

References

1. Arbuckle MA, Reichlin M, Harley JB, James JA (1999) The development of lupus humoral autoimmunity for anti-Sm autoantibodies is consistent with predictable sequential B cell epitope spreading. Scand J Immunol 50:447–455
2. Aya T, Mizuno F, Osato T (1980) Immunologic cytotoxicity against autologous human lymphocytes transformed or infected by Epstein-Bar virus: role of antibody-dependent cellular cytotoxicity in health individuals. J Natl Cancer Inst 65:265–271
3. Evans AS, Rothfield NF, Niederman JC (1971) Raised antibody titres to E.B. virus in systemic lupus erythematosus. Lancet I:167–168
4. Gergely L, Czegledy J, Vaczi L, Gonczol E, Szegedi G, Berenyi E (1973) EBV antibodies in systemic lupus erythematosus. Lancet I:325–326
5. Harley JB, Gaither, KK (1988) Autoantibodies. Rheum Dis Clin North Am 14:43–56
6. Harley JB, James JA (1995) Autoepitopes in lupus. J Lab Clin Med 126:509–516
7. Hochberg MC (1997) Updating the American College of Rheumatology 1997 revised criteria for the classification of systemic lupus erythematosus. Arthritis Rheum 40:1725
8. Incaprera M, Rindi L, Bazzichi A, Garzelli C (1998) Potential role of the Epstein-Barr virus in systemic lupus erythematosus autoimmunity. Clin Exp Rheumatol 16:289–294
9. James JA, Harley JB (1992) Linear epitope mapping of an Sm B/B' polypeptide. J Immunol 148:2074–2079
10. James JA, Kaufman KM, Farris AD, Taylor-Albert E, Lehman TJ, Harley JB (1997) An increased prevalence of Epstein-Barr virus infection in young patients suggests a possible etiology for systemic lupus erythematosus. J Clin Invest 100:3019–3026
11. James JA, Scofield RH, Harley JB (1997) Lupus humoral autoimmunity after short peptide immunization. Ann N Y Acad Sci 815:124–127
12. James JA, Neas BR, Moser KL, Hall T, Bruner GR, Sestak AL, Harley JB (2001) Systemic lupus erythematosus in adults is associated with previous Epstein-Barr virus exposure. Arthritis Rheum 44:1122–1126
13. Kang I, Quan T, Nolasco H, Park SH, Hong MS, Crouch J, Pamer EG, Howe JG, Craft J (2004). Defective control of latent Epstein-Barr virus infection in systemic lupus erythematosus. J Immunol 172:1287–1294
14. Klippel JH, Decker JL (1973) Epstein-Barr virus antibody and lymphocyte tubuloreticular structures in systemic lupus erythematosus. Lancet II:1057–1058
15. Lau CS, Yuen KY, Chan KH, Wong RW (1998) Lack of evidence of active lytic replication of Epstein-Barr and cytomegaloviruses in patients with systemic lupus erythematosus. Chin Med J (Engl) 111:660–665
16. Llorente L, Zou W, Levy Y, Richaud-Patin Y, Wijdenes J, Alcocer-Varela J, et al (1995) Role of interleukin 10 in the B lymphocyte hyperactivity and autoantibody production of human systemic lupus erythematosus. J Exp Med 181:839–844
17. Mahot S, Sergeant A, Drouet E, Grauffat H (2003) A novel function for the Epstein-Barr virus transcription EB1/Zta: induction of transcription of the hIL-10 gene. J Gen Virol 84:965–974
18. Marchini B, Dolcher MP, Sabbatini A, Klein G, Migliorini P (1994) Immune response to different sequences of the EBNA I molecule in Epstein-Barr virus-related disorders and in autoimmune diseases. J Autoimmun 7:179–191
19. Miyashita EM, Yang B, Lam KM, Crawford DH, Thorley-Lawson DA (1995) A novel form of Epstein-Barr virus latency in normal B cells in vivo. Cell 80:593–601
20. Newkirk, MM, Tsoukas C (1992) Effect of ultraviolet irradiation on selected host cell proteins including Ro/SS-A and Epstein-Barr virus in cultured lymphoblastoid cell lines. J Autoimmun 5:511–525
21. Phillips PE, Hirshaut Y (1973) Epstein-Barr virus antibody levels in systemic lupus erythematosus. Arthritis Rheum 16:97–101
22. Saito I, Servenius B, Compton T, Fox RI (1989) Detection of Epstein-Barr virus DNA by polymerase chain reaction in blood and tissue biopsies from patients with Sjogren's syndrome. J Exp Med 169:2191–2198
23. Scofield RH, James JA (1999) Immunization as a model for systemic lupus erythematosus. Semin Arthritis Rheum 29:140–147
24. Tsai YT, Chiang BL, Kao YF, Hsieh KH (1995) Detection of Epstein-Barr virus and cytomegalovirus genome in white blood cells from patients with juvenile rheumatoid arthritis and childhood systemic lupus erythematosus. Int Arch Allergy Immunol 106:235–240

25. Tsokos GC, Magrath IT, Balow JE (1983) Epstein-Barr virus induces normal B cell responses but defective suppressor T cell responses in patients with systemic lupus erythematosus. J Immunol 131:1797–1801
26. Yokochi T, Yanagawa A, Kimura Y, Mizushima YJ (1989) High titer of antibody to the Epstein-Barr virus membrane antigen in sera from patients with rheumatoid arthritis and systemic lupus erythematosus. J Rheumatol 16:1029–1032